Ana Rita Valente Ribeiro
Licenciada em Análises Clínicas e Saúde Pública

Dissecting deregulated cell-to-cell communication in *in vitro* Alzheimer's disease models

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Dora Brites, PhD, Faculdade de Farmácia da Universidade de Lisboa
Co-orientador: Adelaide Fernandes, PhD, Faculdade de Farmácia da Universidade de Lisboa

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UNIVERSIDADE NOVA DE LISBOA

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“A goal without a plan is just a wish”
Antoine de Saint-Exupéry

Para os meus pais

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ABSTRACT

The interplay between neurons and glia cells has recently gained new interest in Alzheimer's disease (AD) pathogenesis. Neuroinflammation is a well-known hallmark, where microglia and astrocytes play a major role. Extracellular vesicles, such as exosomes, are crucial in the interplay between neurons and glial cells, and in AD spreading. However, exosomes may have both beneficial and harmful effects in AD process, thus requiring further clarification. Our first goal was to assess microglia response to exosomes derived from an AD *in vitro* neuronal cell model, using respectively human CHME3 microglia and SH-SY5Y neurons expressing APP Swedish mutation (SH-SY5Y APP_{Swe}). Exosomes were collected by differential centrifugation and incubated with microglia for 24 h to assess microglia direct response. Microglia was then left for an additional 24 h period to evaluate microglia recovery ability. SH-SY5Y APP_{Swe} cells displayed increased levels of miR-155 and miR-21, which were recapitulated in their exosomes. They also showed upregulated levels of alarmins and cytokines, from which two of them (HMGB1 and TNF-α) were not part of exosomal cargo. Exosome-treated microglia revealed an increase in miR-155 and miR-21, as well as in S100B and TNF-α gene expression, together with miR-124 decrease. In addition, uptake of exosomes by microglia led to lysosomal impairment and to a sustained elevation of miR-155 over the 24 h recovery period. Induced pluripotent stem cells (iPSCs) are indicated as a gold model to investigate AD pathology. Such feature, led us to the second goal of evaluating the phenotype of iPSCs-derived astrocytes from AD patients with the PSEN1ΔE9 mutation. AD-astrocytes evidenced a depressed RAGE/miR-155 axis, not recapitulated in their exosomal cargo with decreased levels of HMGB1, TNF-α and S100B genes. In sum, AD-neurons determine microglia activation and dysfunction through their exosomes, and AD-astrocytes display a less reactive phenotype, while releasing exosomes depleted in inflammatory machinery.

Keywords: AD models**;** iPSCs-derived astrocytes; SH-SY5Y APP_{Swe} cells; exosomes; miRNAs; activated/deactivated microglia

RESUMO

A interação entre neurónios e células da glia revelou ser muito importante na patogenicidade da Doença de Alzheimer (DA) e a neuroinflamação um fator de risco, onde a microglia e os astrócitos têm um papel importante. As vesículas extracelulares, como os exosomas, são cruciais na interação entre neurónios e células da glia, bem como na disseminação da DA. Dado que tanto podem ter um efeito benéfico como prejudicial, são necessários estudos que melhor clarifiquem o seu papel. O primeiro objetivo foi avaliar a resposta da microglia humana CHME3 aos exosomas libertados de neurónios SH-SY5Y expressando a mutação *Swedish* (SH-SY5Y APP_{Swe}). Os exosomas foram recolhidos por centrifugação diferencial e incubados com a microglia durante 24h para avaliação dos seus efeitos diretos na célula. A recuperação da microglia foi avaliada após um período adicional de 24h. Encontrámos elevação de miR-155 e miR-21 tanto nas células SH-SY5Y APP_{Swe}, como nos seus exosomas. Relativamente às citoquinas e alarminas, também aumentadas nas células, verificámos que duas delas (HMGB1 e TNF-α) não integravam o conteúdo exosomal. O efeito dos exosomas na microglia traduziu-se num aumento de miR-155 e miR-21, bem como de S100B e TNF-α, e diminuição de miR-124, verificando-se uma falência lisosomal e sobre-expressão de miR-155 após o período de recuperação. As células estaminais pluripotentes induzidas (iPSCs) são consideradas um modelo excelente para investigar a patogenicidade da DA. Deste modo, o segundo objetivo desta tese foi o de avaliar o fenótipo dos astrócitos gerados de iPSCs de doentes com a mutação PSEN1ΔE9. Estes astrócitos revelaram redução de RAGE/miR-155, não observada nos exosomas, onde se encontrou uma diminuição de S100B, HMGB1 e TNF-α. Em suma, os exosomas resultantes de neurónios de DA causam ativação e disfunção da microglia, e os astrócitos provenientes de iPSCs de DA são menos reativos e libertam exosomas empobrecidos em mediadores inflamatórios.

Palavras-chave: Modelos de DA; astrócitos derivados de iPSCs; células SH-SY5Y APP_{Swe}; exosomas; miRNAs; microglia ativada/desativada.

INDEX

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

AB/AM	Antibiotic/antimycotic
AD	Alzheimer's disease
AICD	APP intracellular C-terminal domain
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
Argo	Argonaut protein
Aβ	Amyloid-beta
BACE1	Beta-site APP-cleaving enzyme 1 or β secretase
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenic protein 4
BSA	Bovine serum albumin
Cas9	CRISPR associated protein-9 nuclease
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
COX-2	Cyclooxygenase-2
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
Cx	Connexin
CX3CR1	CX3C chemokine receptor 1
DAMPs	Danger-associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
ERK	Extracellular signaling-related kinase
ESC	Embryonic stem cells
ESCRTs	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
Exp5	Exportin 5
FAD	Familial AD
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	Guanosine diphosphate
GFAP	Glial fibrillary acid protein
GLT-1	Glutamate tansporter-1
GTP	Guanosine triphosphate
HD	Huntington's disease
hiPSCs	human induced Pluripotent Stem Cells
HMGB1	High-mobility group box 1
IFN-γ	Interferon-γ
IL	Interleukin
ILVs	Intra-luminal vesicles
iNOS	Inducible nitric oxide synthase
IPSCs	induced Pluripotent Stem Cells
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	*c*-Jun N-terminal kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
L-glu	L-glutamine
LPS	Lipopolysaccharide
MCI	Mild Cognitive Impairment
miR	MicroRNA
miRNAs	MicroRNAs
mRNA	Messenger RNA
MVBs	Multivesicular bodies
NEAA	Non-essential amino acid
NEP	Neprilysin
NDM	Neural differentiation medium
NF-κB	Nuclear factor-kappa B
NFTs	Neurofibrillary tangles
NMAD	N-methly-D-aspartatic acid receptor
NO	Nitric oxide
NPCs	Neural Progenitor Cells
NTA	Nanoparticle Tracking Analysis
p38/MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PKC	Protein kinase C
PSEN1	Presenilin 1
PSEN2	Presenilin 2
p-tau	Hyperphosphorylated tau

qPCR	quantitative polimerase chain reaction
qRT-PCR	quantitative real time-PCR
RA	Retinoic acid
RAGE	Receptor for advanced glycation end-products
RISC	RNA-induced silencing complex
RT	Room temperature
S100B	S100 calcium-binding protein B
SAD	Sporadic AD
SCI	Spinal cord injury
SH-SY5Y APP_{Swe}	SH-SY5Y expressing APP_{695} Swedish mutation
SOCS-1	cytokine signaling-1
SOD1	Superoxide dismutase 1
TGF-β	Transforming growth factor-β
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TREM-2	Triggering receptor expressed on myeloid cells-2
ULA	Ultra-low attachment
wt	Wild type

I. Introduction

1. Alzheimer's disease

Initially described in 1906 by Alois Alzheimer, Alzheimer's disease (AD) is the most common, progressive and irreversible neurodegenerative disorder, characterized by memory loss, cognitive impairment and behavioral abnormalities. AD is the leading cause of dementia in the elderly, accounting for more than 80% of cases worldwide. Currently, it is estimated that the disease affects 37 million cases (WHO, 2017) and, by the year of 2050, it is expected to achieve 13.8 million cases, with nearly a million new cases per year (Kumar et al., 2015). Curiously, a recent report compared dementia prevalence among 65 years individuals or older, between 2000 and 2012, showing that a reduction from 11.6% to 8.8% has occurred. This decreased prevalence was associated to increased educational attainment and healthier lifestyles (Langa et al., 2016).

1.1. Alzheimer's disease etiology

The etiology of AD remains unclear. Apparently it is likely to be the combination of both environmental and genetic factors, in which older age is the strongest risk factor (Mayeux and Stern, 2006). According to the age of onset, there are two types of AD: early onset or familial AD (FAD < 65 years) and late onset or sporadic AD (SAD > 65 years). FAD is associated with a rapid progression rate and is often caused by a rare autosomal dominant mutation in genes for amyloid precursor protein (APP) or associated with its processing, such as Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2). On the other hand, SAD is associated with late symptoms appearance even though the main cause is still unclear (Dzamba et al., 2016). Polymorphisms in Apolipoprotein E (Apo E), particularly in the Apo E ε4 allele, has been considered an important risk factor across many studies (Qiu et al., 2009). Apo E has an important role in the amyloid-beta (Aβ) peptide metabolism suggesting that it affects Aβ deposition forming senile plaques (Kim et al., 2009). This evidence is supported by studies comparing Apo E ε4 carries with non-carries (Liu et al., 2013; Yamazaki et al., 2016). Recent genetic studies have identified a rare variant of the triggering receptor expressed on myeloid cells-2 (TREM-2) as an important risk factor for AD (Colonna and Wang, 2016). TREM-2 is a transmembranar receptor and is found in various tissue macrophages, including in the central nervous system (CNS) microglia. It has an important role on the Aβ clearance and phagocytosis, and in the presence of mutations leads to an increase of Aβ accumulation, as well as to an inflammatory reaction (Hickman and El Khoury, 2014).

1.2. Alzheimer's disease symptoms and treatment

AD symptoms start with manifest symptoms related to recent memory and thinking ability called Mild Cognitive Impairment (MCI). Only 10-15% of people diagnosed with MCI develop AD (Alberdi et al., 2016), but the reason why some people do not develop dementia is still an unexplored field. The final AD stage is characterized by memory, thinking and behavioral impairments affecting patient's ability in daily life. Neurobiological investigations have shown a reduction in the number of cholinergic neurons

together with synapse loss, mostly in the hippocampus, entorhinal and frontal cortices, which are involved in cognitive functions like memory and language, and at the amygdala, prefrontal cortex and hypothalamus, related with emotional behavior (Whitehouse et al., 1982).

Despite problem symptoms, an accurate diagnosis of AD remains difficult to establish. Cognitive impairment in older people is frequently due to the existence of co-morbidities (Alves et al., 2012). In contrast, being AD a progressively condition, a proper diagnosis can only be achieved on an advanced stage of neurodegeneration (Kidd, 2008). A definitive diagnosis is based on the clinical and pathological hallmarks, as senile plaques and neurofibrillary tangles discussed later in this thesis. Nowadays it is already possible to identify the formation of senile plaques in patients using the Pittsburg Compound-B (PiB)-PET imaging (Cohen et al., 2012), but the clinical diagnosis realized by highly experienced and sensitive clinicians remains the most practicable approach (Alves et al., 2012). Moreover, physiological, biochemical and anatomic biomarkers, measurable *in vivo*, can also be considered to improve AD diagnosis (Jack and Holtzman, 2013). Aβ deposits, together with total and phosphorylated tau, are measurable in the cerebrospinal fluid (CSF). Additionally, extracellular vesicles (EVs), such as exosomes, containing Aβ peptide deposits can be isolated from body fluids (e.g. serum and CSF), further suggesting its utility as a novel non-invasive strategy in AD diagnosis (Vella et al., 2016). Apart from AD hallmarks content, exosomes can also carry microRNAs (miRNAs) involved in AD pathogenesis suggesting another useful and complementary tool in AD diagnosis (Gallo et al., 2012; Vella et al., 2016) Regarding the possibility of using common biomarkers to diagnose AD, usually they are only used to confirm or exclude other clinical symptoms related with neurodegenerative dementia (Leidinger et al., 2013).

Due to AD multifactorial etiology, a reliable and effective therapy that would reverse its progression has been extremely difficult to develop. Nowadays, symptom treatment is the only practicable approach to improve patient's life quality (Khanam et al., 2016). Drugs approved by Food and Drug Administration (FDA) to AD include acetylcholinesterase inhibitors (Donepezil, Galantamine and Rivastigmine), non-competitive N-methly-D-aspartatic acid receptor (NMAD) antagonist (Memantine), and behavior signs adjuvants (antipsychotics and anticonvulsants) (Scarpini et al., 2003). In an attempt to develop new effective treatments, several studies are in progress, with some including new pharmacological compounds and innovative immunotherapy strategies against Aβ aggregates (Alves et al., 2012).

1.3. Pathological hallmarks: amyloid-beta peptide and neurofibrillary tangles

Histopathological hallmarks are represented by extracellular accumulations of misfolded Aβ peptide, known as senile or neuritic plaques, and intracellular accumulations of hyperphosphorylated tau (p-tau) protein in neurofibrillary tangles (NFTs) and neuropil threads (Rosenberger et al., 2016)**,** in the autopsied brains of people with AD. Alterations in the metabolism of the amyloid precursor protein (APP) and tau hyperphosphorylation are two hallmarks that are believed to play a key role in AD genesis (Figure I.1). Additional changes include chronic neuroinflammation, neuronal loss and brain atrophy, as mentioned above.

The central pathological feature in AD is the deposition of Aβ peptides. APP is a membrane glycoprotein synthetized in the endoplasmic reticulum and present in the brain and CNS. Physiologically,

APP can be processed by two main pathways: non-amyloidogenic and amyloidogenic, in which APP is sequentially cleaved by proteolytic enzymes, named secretases (Gupta and Goyal, 2016) (Figure I.1). In the non-amyloidogenic pathway, the extracellular APP domain is cleaved by α-secretase forming a soluble extracellular fragment called sAPPα. Subsequently, a plasma protein named γ-secretase, composed by the anterior pharynx defective 1, nicastrin, PSEN 1, PSEN 2 and PSEN enhancer 2, forms an intracellular fragment called APP intracellular C-terminal domain (AICD) (Murphy and LeVine, 2010). In contrast, in the amyloidogenic pathway, the extracellular APP domain is cleaved by β-secretase (beta-site APP-cleaving enzyme 1 - BACE1), releasing the sAPPβ fragment. Afterwards, the remaining fragment C99 is processed by γ-secretase forming two Aβ major isoforms of different length, $A\beta_{40}$ with 40 amino acids in length, and $A\beta_{42}$, with 42 amino acids in length (Bolduc et al., 2016). Physiologically, there is a balance between production and clearance of Aβ peptides. However, in pathological conditions there is an increase of total Aβ concentration or $A\beta_{40}/A\beta_{42}$ ratio or even a decrease in the clearance of Aβ leading to elevated levels of $A\beta_{42}$ (Gupta and Goyal, 2016). $A\beta_{42}$ isoform is considered more hydrophobic and aggregates faster, thus resulting in the formation of neurotoxic senile plaques (Peric and Annaert, 2015).

FAD is associated with several mutations in genes related to APP processing, as stated previously. Among them, the Swedish mutation was first described in 1992, when Mullan and colleagues observed two Swedish families linked by genealogy (Mullan et al., 1992). It is characterized as a double mutation in exon 16 at codons 670 and 671 (K670M/N671L) (Marques et al., 2003) leading to a new cleavage site in APP that favors the amyloidogenic pathway increasing the total amount of Aβ and its further accumulation.

In addition to Aβ plaques, NFTs composed by p-tau protein are the other hallmark associated with AD. Tau protein, in normal conditions, is associated to the microtubules, stabilizing cell cytoskeleton. P-tau sequesters normal tau away from microtubules endangering the axonal transport in affected brain regions (Figure I.1). Consequently, neurons develop NFTs, synaptic dysfunction, oxidative stress, mitochondrial impairment and DNA damage causing neuronal loss and microglia activation (Qin et al., 2016). Traditionally, AD has been considered a disorder proceeding with a dual pathway as described above. However, recent studies have shown functional interactions between Aβ and tau protein. An *in vitro* study using SH-SY5Y neuroblastoma cell line, revealed that when cells were cultured in a media supplemented with $A\beta_{42}$ (and not $A\beta_{40}$), tau phosphorylation was increased (Han and Shi, 2016). Moreover, pathological phosphorylation might be mediated by activation of protein kinases dependent on Aβ, particularly GSK3 (Tatarnikova et al., 1800). Therefore, synergic interactions between these two toxic proteins accelerate AD pathogenesis

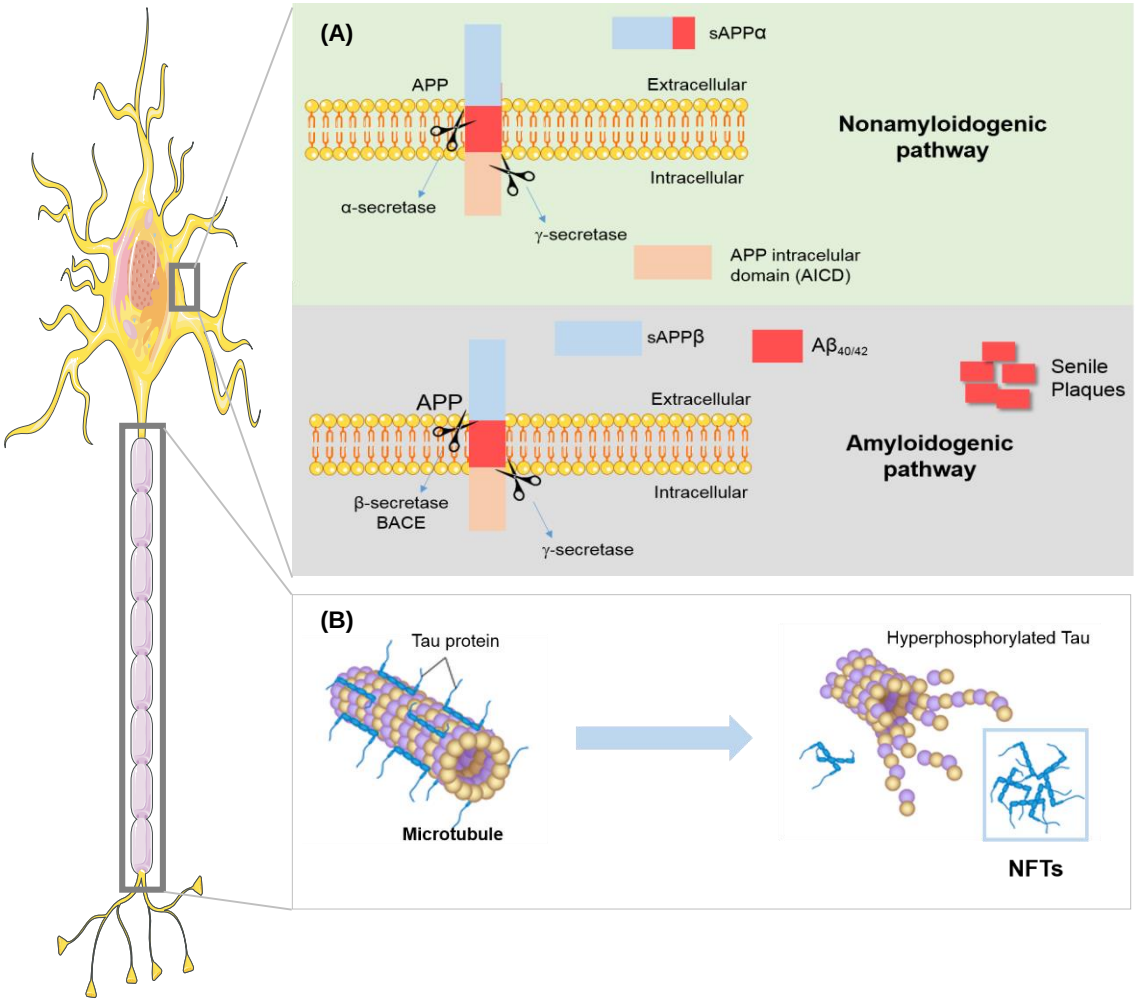

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Figure I.1-Alzheimer's disease pathological hallmarks. (A) Changes in the metabolism of the amyloid precursor protein (APP). Most APP is processed through the non-amyloidogenic pathway, in which cleavage by α-secretase generates sAPPα soluble fragment and, subsequently, cleavage by γ-secretase forms an intracellular fragment called APP intracellular C-terminal domain (AICD). In the amyloidogenic pathway, APP is cleaved by β-secretase releasing sAPPβ fragment and then, the remaining APP fragment, is cleaved by γ-secretase leading to the formation of the amyloid-beta (Aβ) peptide. In the end, mediated by Aβ accumulation, there is the formation of high complex molecules known as senile plaques. **(B) Tau hyperphosphorylation:** tau protein is normally associated to the microtubules, thus stabilizing cell cytoskeleton. In Alzheimer's disease (AD), tau suffers hyperphosphorylation and consequently detach from the microtubules with the formation of neurofibrillary tangles (NFTs). NFTs contribute to axonal transport impairment and neuronal dysfunction.

2. Cellular diversity of central nervous system: the interaction between neurons and glial cells

The CNS exhibits a tremendous cell type diversity. Apart from neurons, glial cells constitute a large fraction, between 33 and 66%, of the mammalian brain. Glial cells were first identified by Rudolf Virchow, Santiago Ramón y Cajal and Pío del Río-Hortega and suggested that they solely function as so-called "nerve glue". However, with time, scientist started to speculate about additional possible roles for these cells (Jäkel and Dimou, 2017). In fact, nowadays, glial cells are intended to be more than glue due to their involvement in many central hemostatic processes and also during development. Although many studies have been performed in order to understand glia specific roles, the full properties of these cells

remain unresolved (Jäkel and Dimou, 2017). There are three types of glial cells, microglia, astrocytes and oligodendrocytes, in each one play specific roles in SNC.

Oligodendrocytes are the myelinating cells of the CNS and constitute about 5 to 10% of the total glial population. These cells are responsible for myelin production around axons, which is essential not only for the rapid and efficient conduction of the electrical impulses along the axons, but also for preserving axonal integrity (Barateiro et al., 2017). Regarding AD pathogenesis, some *in vitro* studies have shown that oligodendrocytes and myelin alterations occurs before the appearance of Aβ and tau pathology (Cai and Xiao, 2016). In fact, it has been suggested that myelin breakdown releases iron thus promoting the development of toxic Aβ fibrils and enhancing the formation of senile plaques (Bartzokis et al., 2007).

Microglia cells are distributed throughout the CNS and present multiple heterogeneous roles within the healthy CNS, showing diverse morphological and functional profiles, depending on their surrounding environment (Nimmerjahn et al., 2005). They are dynamic cells responsible for the surveillance of the extracellular space working as protective cells (Caldeira et al., 2014). Considered as mononuclear phagocytes, or even the CNS macrophages (Dzamba et al., 2016), the fact of sharing phenotypic characteristics and lineage-related properties with bone-marrow-derived macrophages, makes microglia capable of secreting cytokines and serving as antigen-presenting cells (Harry and Kraft, 2008), although not having the same cell origin. In the presence of diverse types of damage and stimulus, microglia becomes activated, changing their morphology from ramified to amoeboid, migrating to the lesion sites and clearing the debris of dead cells and pathogens (Brites and Vaz, 2014). During this process, microglia releases not only neurotrophic factors, but also inflammation-related molecules (e.g. pro-inflammatory cytokines and neurotoxic molecules) leading to a state of neuroinflammation (Pinto et al., 2017).

Lastly, astrocytes are the most abundant non-neural cells and are predominantly responsible for maintaining a proper chemical environment for neuronal signaling and for providing metabolic connections through the blood-brain barrier (BBB) (Purves et al., 2001). Moreover, astrocytes express metabotropic and ionotropic receptors capable of sensing neuronal activity. They express glutamate, GABA and glycine receptors involved in the tripartite synapse with pre- and post-synaptic neurons (Meyer and Kaspar, 2016). Therefore, the interplay between structure, morphology and functional characteristics allow them to exert an active influence in the neuronal signaling and protection by releasing antioxidant molecules, such as glutathione, thus preventing neurons from oxidative stress. In addition, by uptaking excess of glutamate they prevent its excitotoxicity (Osborn et al., 2015).

Over the past two decades, microglia and astrocytes have gained special attention in what concerns AD impairment, since clear evidences of their involvement have been reported in several neurodegenerative disorders (Meyer and Kaspar, 2016). Their role in AD pathogenesis will be further discussed and elucidated in this chapter.

3. Alzheimer's disease as a non-cell autonomous disease

3.1. Vesicular trafficking mediated by exosomes and microvesicles

Eukaryotic cells maintain contact with the extracellular compartment by receiving molecular signals (e.g. cytokines and chemokines) and by secreting proteins into the extracellular space. For

communication, each cell has a complex network of membranes that allows them to uptake macromolecules, named endocytosis, and release biomolecules to the exterior environment, named exocytosis (Keller et al., 2006). There are three main vesicles described so far: (i) microvesicles or ectosomes (150 nm to 1 µm), which directly bud from the plasma membrane, (ii) apoptotic bodies (50-500 nm) that are released by apoptotic cells, and (iii) exosomes (40–100 nm) that derive from multivesicular bodies (MVBs) in a mechanism described next (Urbanelli et al., 2013).

Exosomes are small vesicles that are secreted from the majority of cell types, including neurons, oligodendrocytes, microglia and astrocytes (Yuyama et al., 2014) and are retrieved *in vivo* in several body fluids, such as plasma, urine, saliva, seminal fluid, amniotic liquid, ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk and CSF (Urbanelli et al., 2013). Exosomes are endocytic membrane-derived vesicles that are contained in MVBs in the endosomal system and secreted upon MVB fusion with the plasma membrane (Brites and Fernandes, 2015). In other words, these small vesicles have their own cytosol and are secreted by exocytosis from the MVBs, as shown in Figure I.2. MVBs result from inward budding inside an intracellular endosome forming intra-luminal vesicles (ILVs), which progressively accumulate inside lumen of the late endosome (Kowal et al., 2014). Endosomal sorting complexes required for transport (ESCRTs) and ubiquitination are involved in the biogenesis and degradation mechanisms of MVBs (Cocucci and Meldolesi, 2015).

Besides their morphology, exosomes have a particular composition due to their endosomal origin (Figure I.2). All exosomes contain membrane transporter and fusion proteins, including GTPases, annexins and flotillin, clusters of differentiation (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70, Hsp90), proteins involved in MVB biogenesis (Alix, TSG101), cytoskeleton proteins, lipids, phospholipids and saccharide groups. Exosomes also exhibit a few markers from other intracellular organelles (Golgi complex, mitochondria, nucleus) proving to be a specific subcellular compartment (Kowal et al., 2014). Although these proteins are used as positive exosomes markers, there is wide variation across exosomes from different sources (Vlassov et al., 2012). Furthermore, exosomes have been reported to contain significant amounts of microRNAs (miRNAs), other non-coding RNAs, as well as messenger RNA (mRNA) (Vlassov et al., 2012). Several papers indicate that the exosomes RNA cargo is significantly different from the origin cell content. This counteracts several papers related to cancer studies, which have noted that the miRNAs content for their originating cells is similar to that found in circulating exosomes (Rabinowits et al., 2009). Regarding this, authors have postulated the feasibility of using exosomes as a basis for diagnostic marker (Vlassov et al., 2012).

Ectosomes, also called microvesicles, are quite large vesicles that bud directly from the plasma membrane (Figure I.2) (Brites and Fernandes, 2015) and are released into the extracellular space. Shedding of ectosomes often involves a budding process, in which surface pimples selectively accumulate cellular constituents that are packaged into microvesicles (Turola et al., 2012). Regulation of this process involves several enzymes such as calpain, flippase, floppase, scramblase and gelsolin (Mathivanan et al., 2010). Due to their formation process, ectosomes contain a variety of cell surface

receptors, intracellular signaling proteins and genetic material derived from the cell of origin (Turola et al., 2012).

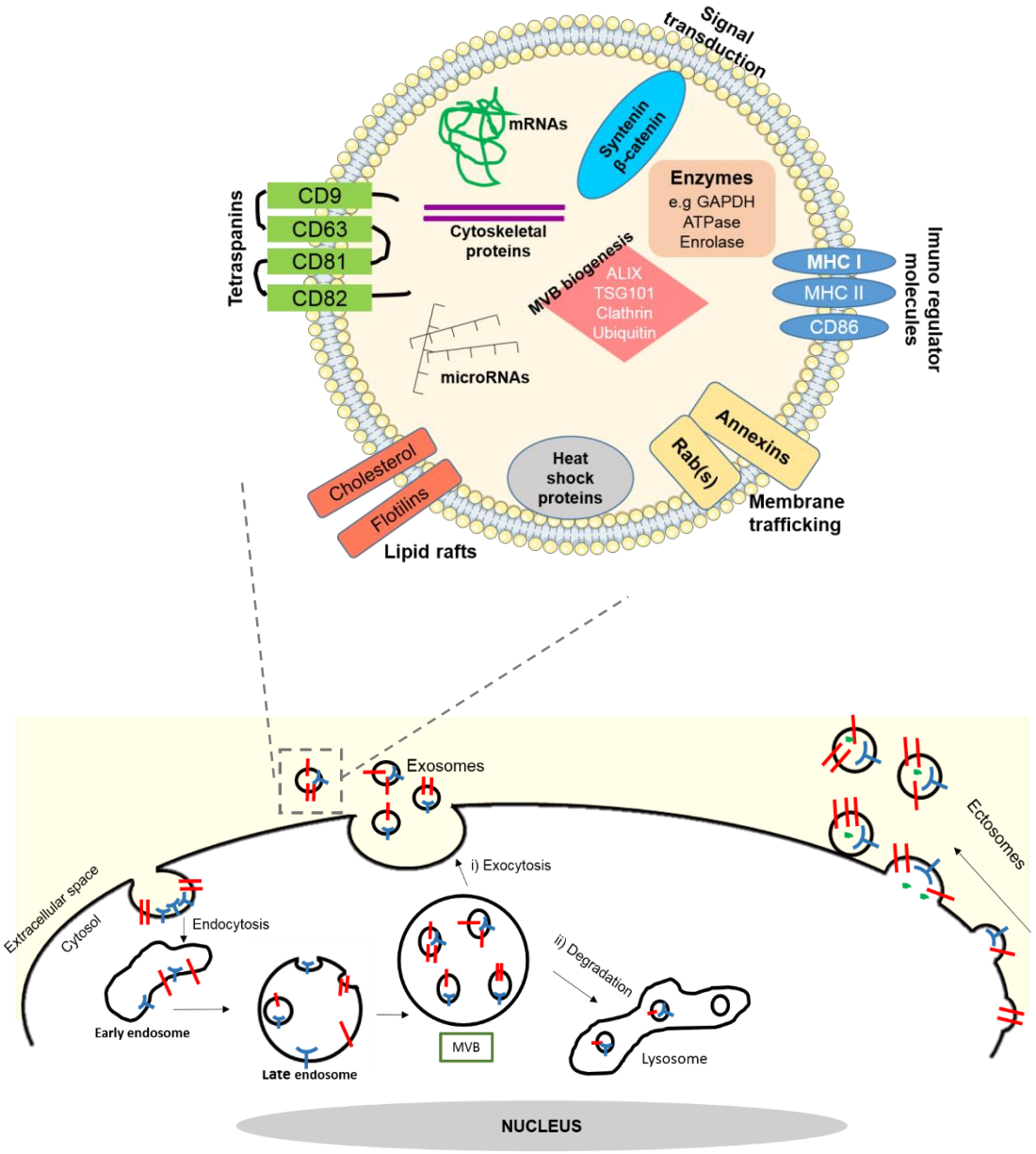

Figure I.2-Biological characteristics of exosomes: biogenesis, composition and cargo. Vesicular trafficking and cell communication are mediated by exosomes and ectosomes. Ectosomes are generated by direct budding of the plasma membrane. Exosomes are produced by exocytosis from multivesicular bodies (MVBs), in which they are produced by inward budding of the plasma membrane originating, consequently, the early and late endosome. MBVs can be i) released into the extracellular space as exosomes or ii) degraded via lysosomes. Exosome composition is mainly determined by their endosomal origin. Exosomal membranes are composed by tetraspanins, lipid rafts, proteins involved in membrane trafficking and immuno regulator molecules. Several types of cytosolic proteins and nucleic acids may be identified inside the lumen of exosomes. Cytosolic proteins include heat shock proteins, cytoskeletal proteins, enzymes, proteins involved in MVBs biogenesis and proteins involved in signal transduction. Some nucleic acids already identified in exosomes are messenger RNAs (mRNAs) and microRNAs (miRNAs).

3.2. Neuron and glia cells interplay: cell-to-cell communication

Intercellular communication can be mediated through direct cell-to-cell communication or by the action of secreted molecules (Frühbeis et al., 2013).

As previously stated, eukaryotic cells use EVs to communicate and exchange information between cells (Figure I.3). EVs can interact with the recipient cells by three mechanisms: (i) EVs membrane proteins bind directly to the signaling receptors of target cells; (ii) EVs fuse with the plasma membrane and release their cargo inside recipient cell; and (iii) EVs are internalized into the recipient cells and have two fates (Zhang et al., 2015). In one case, some engulfed exosomes merge into endosomes and undergo transcytosis, which will move exosomes across the recipient cells and release them into neighboring cells. In the other case, endosomes will move to lysosomes and undergo degradation (Zhang et al., 2015). The communication between neurons and glia is essential to synchronize diverse functions with the brain activity. Secreted exosomes by neurons have been implicated in the synapse plasticity. Cultures of cortical neurons with enhanced glutamatergic activity show that the secreted exosomes carrying the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) may help to adapt the efficacy of synaptic transmission by depletion of neurotransmitter receptors from the postsynaptic compartment (Frühbeis et al., 2012). EVs have been suggested as potential carriers in the intercellular delivery of pathological proteins such as misfolded proteins associated to neurodegenerative disorders. This includes p-tau and Aβ in AD, α-synuclein in Parkinson's disease (PD), superoxide dismutase 1 (SOD1) in Amyotrophic lateral sclerosis (ALS) and huntington in Huntington's disease (HD). Concerning AD, studies using *in vivo* and *in vitro* models suggest a role of EVs on Aβ aggregation and neurotoxicity and, on the other side, opening the possibility of their potential in AD therapy, as discussed in Joshi review paper (Joshi et al., 2015). Exosomes delivered by neuroblastoma cells are able to carry Aβ since Ranjendran observed that typical exosomal proteins, such as Alix, appears surrounding the senile plaques (Rajendran et al., 2006) and moreover, subsequent studies *in vivo* have demonstrated that exosomes are specially enriched with APP C-terminal fragments, a source of Aβ (Perez-Gonzalez et al., 2012). Corroborating this, in a recent paper from Yuyama and Igarashi, it is mentioned in, an *in vivo* APP/PSEN 1 transgenic mice model, that intracellular Aβ accumulates in abnormal endosomes, including MVBs, whose ILVs are precursors of exosomes (Yuyama and Igarashi, 2017). In the same article, authors describe that glycosphingolipids present on the outer layer of cellular and exosome membrane are implicated in Aβ binding (Yuyama and Igarashi, 2017). It is important to note that with those studies it was suggested that neurons have the capacity to encapsulate Aβ through exosomes followed by its release into the extracellular medium. Such evidence suggests that exosomes could be a way to neurons get rid of excessive Aβ. Through exosome secretion extracellular Aβ levels raises up and promotes its aggregation. (Joshi et al., 2015). However, regarding discussed evidences, it is still not clear whether exosomes help cells to get rid of potential detrimental components or act as a way of spreading the neurodegenerative "seeds" to other cells (Frühbeis et al., 2012).

Similar to neurons, astrocytes and microglia are able to release EVs to the extracellular space acting in physiological and pathological functions (Frühbeis et al., 2013). On one hand, exosomes delivered by astrocytes are implicated in neuroprotection carrying Hsp/ Hsc70 and synapsin I (Taylor et al., 2007;

Wang et al., 2011), as well as in angiogenesis modulation (Proia et al., 2008). One the other hand, they mediate the propagation of pathogenic proteins. Wang and colleagues described that astrocytes exposed to Aβ are capable of releasing pro-apoptotic exosomes, which in turn suffer uptake by other astrocytes promoting their apoptosis (Wang et al., 2012). In addition, Goetzl and colleagues compared exosomes from astrocytes with those from neurons from plasma samples of AD patients. It was found that levels of BACE1, γ-secretase, sAPPα and sAPPβ were higher in exosomes released by astrocytes. Moreover, amyloid aggregates were not observed in the astrocyte-derived exosomes suggesting that astrocytes are more efficient in Aβ clearance. Therefore, these findings suggest that if the interplay between neurons and astrocytes is a central pathogenic pathway in AD, exosomes from astrocytes may be a valuable window for further research on the neural cell-to-cell interaction (Goetzl et al., 2016).

In what concerns exosomes released by microglia, they may act as vehicles for antigen presentation, cytokines and miRNAs secretion. Back in 2015, in a study conducted by Hirohide and colleagues, it was hypothesized that microglia may facilitate tau protein propagation between neurons by phagocytosing and exocytosing tau protein. Indeed, results have demonstrated that the depletion of microglia dramatically suppressed tau protein propagation, as well as that the inhibition of exosome synthesis significantly reduced its spreading, either *in vitro* or *in vivo* assays (Asai et al., 2015). Microglia-derived EVs also represent a way for microglia to eliminate neurotoxic Aβ when microglial degradative pathways are saturated in response to excessive phagocytosis of amyloid aggregates. Such EVs may then also contribute to the spread and seeding of neurotoxic amyloids in the brain. Increased levels of microglial EVs formation were observed in AD patients and correlated with classical markers of neurodegeneration associated with neuronal damage in the human brain (Agosta et al., 2014).

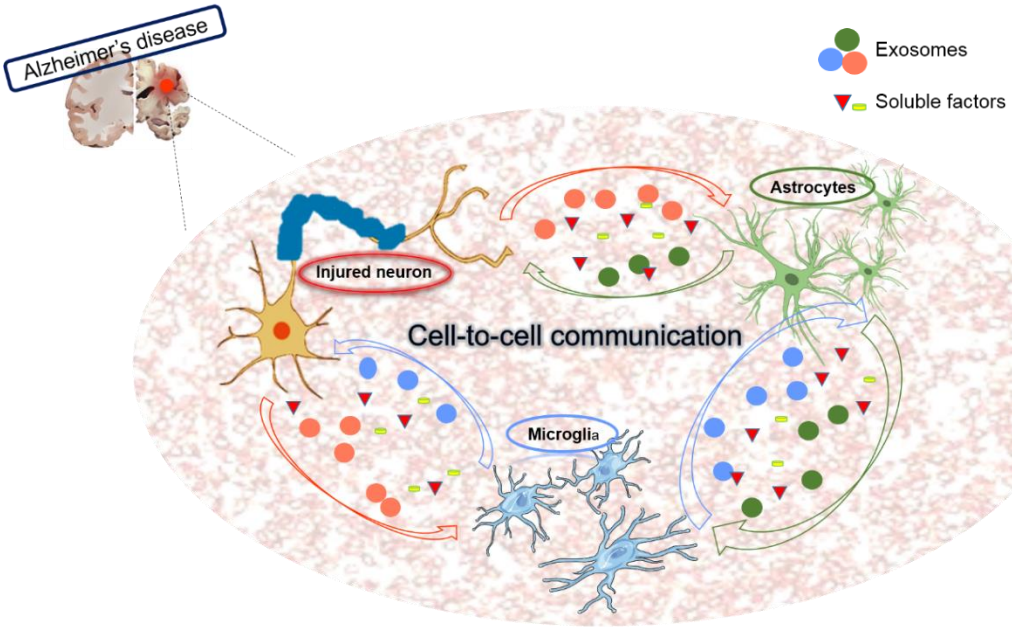

Figure I.3-Cell-to-cell communication between neurons and glial cells through exosomes and soluble factors in Alzheimer's disease. Eukaryotic cells, including neurons and glial cells, give and receive messages from their environment proving that cells do not live isolated. In the context of Alzheimer's disease (AD), damage neurons release exosomes containing pathological features, such as amyloid-beta (Aβ) peptide, hyperphosphorylated tau (p-tau) or even inflammatory cytokines. Microglia and astrocytes, once triggered by delivered exosomes, suffer activation changing their phenotypes. The communication can also be mediated by soluble factors, such as pro- and anti-inflammatory cytokines.

4. Neuroinflammation as an inducer of Alzheimer's disease

Neuroinflammation processes are a central feature of AD and derive from the local activation of innate immune response (Rosenberger et al., 2016). This is a complex process that involves different cellular components in the CNS, such as microglia, astrocytes, ependymal cells, macrophages and mast cells (Figure I.4). Nonetheless, it is still not clear whether inflammation is a simple "watcher", i.e. a consequence or a cause of neurodegeneration (Dá Mesquita et al., 2016).

Receptors binding cytokines on the surface of astrocytes and microglia stimulate a variety of intracellular signaling, implicated in AD pathology, including activation of protein kinase C (PKC), *c*-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38/MAPK), PI3 kinase, extracellular signaling-related kinase (ERK) and caspases 1 and 3 (Garwood et al., 2011).

4.1. Reactive microglia

Similar to macrophages, microglia constitute the first line of defense in the CNS sensing the neuronal environment against pathogens and host-derived ligands (Solito and Sastre, 2012). Moreover, microglia plays an important role in the inflammation resolution.

Many efforts have been done to clarify the role of microglia in what concerns inflammation and AD pathogenesis. Simultaneously with the appearance of amyloid plaques in the brain, there is a dramatic phenotype activation of the surrounding microglia, which displays high immunoreactivity. Post-mortem brain, as well as brain from transgenic APP animals, exhibit increased levels of pro-inflammatory cytokines and chemokines, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 (Heneka and Banion, 2007). Additionally, IFN-γ and TNF-α, not only have toxic effects on neurons, but also contribute to the reduction of insulin degrading enzyme involved in Aβ proteolysis and to the reduction of microglia ability to clean Aβ deposits. This mechanism is described as a secondary mechanism by which inflammation increases amyloid deposition (Mandrekar and Landreth, 2013).

Under pathological conditions, microglia assumes high plasticity and adopts distinct phenotypes (Perry et al., 2010). Indeed, in case of prolonged or chronic stimulation, microglia may become deleterious to the neuronal population.

The surveillance/nonpolarized stage, defined as M0, describes an alert and non-activated microglia stage in which the almost exclusively microglial fractalkine receptor, CX3C chemokine receptor 1 (CX3CR1), is highly expressed (Cunha et al., 2016). Microglia polarization M1, is mediated by activation of toll-like receptors (TLRs) or by IFN-γ, with the production of pro-inflammatory mediators, such as IL-1β, IL-4, TNF-α and transforming growth factor-β (TGF-β) (Figure I.4). Microglia can also assume a M2 phenotype when activated by IL-4 or IL-13, more associated with an anti-inflammatory state or when mixed populations of both types exist (Cameron and Landreth, 2010). Furthermore, in terms of morphology, microglia dramatically change from ramified cells to activated amoeboid cells (Kreutzberg, 1996). However, numerous studies have shown microglial activation phenotypes to be heterogeneous and the categorization as M1 and M2 is still a matter of debate.

4.2. Reactive astrocytes

Astrocytes are crucial regulators and depending on the context and time, they may either promote immunosuppression and tissue repair mediated by anti-inflammatory molecules (e.g. TGF-β), or exacerbate inflammation and tissue damage (Colombo and Farina, 2016). This is corroborated by membrane expression of TLRs, being TLR3 predominantly expressed (Chen et al., 2012). TLR3 engagement triggers the production of pro-inflammatory cytokines capable of promoting inflammatory responses, such as TNF-α, IL-6 and inducible nitric oxide synthase (iNOS), which leads to nitric oxide (NO) increase. Indeed, high levels of such molecules were found in serum and brain of AD patients, compared to non-AD patients, and might exert a direct neurotoxic effect (Calsolaro and Edison, 2016). Despite cytokine toxic effect, they can also upregulate β-secretase mRNA expression and consequently BACE1 enzymatic activity, which in turn is a key regulator of Aβ formation (Chen et al., 2012). Aβ production seems to be increased not only because of cytokine action, but also by TNF-α activated nuclear factor kappa B (NF- κB) signal (Chen et al., 2012) and by upregulation of critical inflammatory mediators, such as TNF-α, IL-1β and cyclooxygenase-2 (COX-2) (Figure I.4) (Medeiros and LaFerla, 2013). Extended exposure to these cytokines compromises the integrity of astrocytes and BBB composition (Minter et al., 2016). Due to their morphology, astrocytes extend thin branches allowing contact with neuronal cells bodies, dendrites and synapse terminals. Spatial contact between neurons and astrocytes allows astrocytes to sense neuronal activity (Meyer and Kaspar, 2016).

Reactive astrogliosis is currently accepted (Rodríguez-Arellano et al., 2016) as a defensive process associated with gradated continuum morphological, molecular and functional changes (Sofroniew and Vinters, 2010) in response to various signals in the extracellular space. Such signals comprehend bacterial molecules [e.g. lipopolysaccharide (LPS)], misfolded proteins and protein aggregates (e.g. Aβ), increase of cytokines [e.g. IL-6, ciliary neurotrophic factor (CNTF), TNF-α, INFγ, IL-1, IL-10, TGF-β, basic fibroblast growth factor (bFGF) 2, among others] and chemokines, or the absence of normal signals from surrounding cells, like neurotransmitters and growth factors (Haim et al., 2015). Astrogliosis results in astrocytes hypertrophy, upregulated expression of intermediated filaments like, predominantly, glial fibrillary acid protein (GFAP) and vimentin, S100 calcium binding protein B (S100B) and astrogial connexins (Cx30 and Cx43) (Yi et al., 2016), and production of inflammatory factors (cytokines, chemokines and growth factors) (Dzamba et al., 2016). GFAP is widely used, both in *in vivo* and *in vitro* studies, for identification of astrogliosis (Colombo and Farina, 2016).

In AD, astrogliosis has an important role in the pathological progression. Senile plaques and p-tau are usually surrounded by activated astrocytes, wherein this process has thought to be a neuroprotective barrier by restricting them from the rest of brain tissue. In addition, astrocytes are capable to take up Aβ peptides, through the receptor of advanced glycation end product (RAGE), for lysosomal degradation with the purpose of maintaining Aβ homeostasis (Steardo et al., 2015). However, the persistent activation and inflammation may also favor the AD progression (Osborn et al., 2015).

4.3. Danger-associated molecular patterns: the alarmins of neuroinflammation

Danger-associated molecular patterns (DAMPs), also known as alarmins, are a pleiotropic group of intracellular proteins that include, among others, the High-mobility group box 1 protein (HMGB1 or

amphoterin) and calcium binding proteins family known as S100. Under pathological conditions, such as chronic inflammation, DAMPs released into the extracellular space are considered as "danger signals" triggering the activation of the receptor RAGE (Buhimschi et al., 2009), as shown in Figure I.4.

RAGE is a transmembranar receptor, acting as a chief for products of nonenzymatic glycoxidation, HMGB1 and S100 family proteins, as well as Aβ, as described above. Binding of DAMPs to the RAGE intracellular domain, results in the activation of NF-κB and the recruitment of inflammatory cells, which in turn amplify the process of tissue damage (Chavakis et al., 2004).

HMGB1 is primarily located in the nucleus of the majority of the cells. It was originally identified as a non-histone binding DNA protein involved in maintaining DNA structure and regulating gene transcription, among other physiological functions (Frank et al., 2015). Under pathological conditions, HMGB1 is released by the cell into the extracellular space. In the brain, HMGB1 is actively released by activated microglia and passively released by necrotic or damaged cells (Fonken et al., 2016). Consequently, it interacts with TLR2 and TLR4, and with RAGE, as previously mentioned. Thereby, the interaction between HMGB1 and the receptors drives the pro-inflammatory cellular responses (Fonken et al., 2016).

S100B is a member of the S100 family and is mostly expressed by astrocytes. Intracellularly, S100B promotes neuronal proliferation, oligodendrocyte differentiation, astrocyte morphology maintenance, and facilitates the cell migration of both astrocytes and microglia. Extracellularly, S100B can act either as neurotrophic or neurotoxic molecule, depending on the concentration achieved (Barateiro et al., 2016). Nowadays, S100B is considered a peripheral biomarker of brain damage since its level may increase in CSF and/or in blood by several brain pathologies, including astrocytes damage. In what concerns AD, it was reported an association between the deposition of Aβ and the presence of activated astrocytes overexpressing S100B (Chaves et al., 2010).

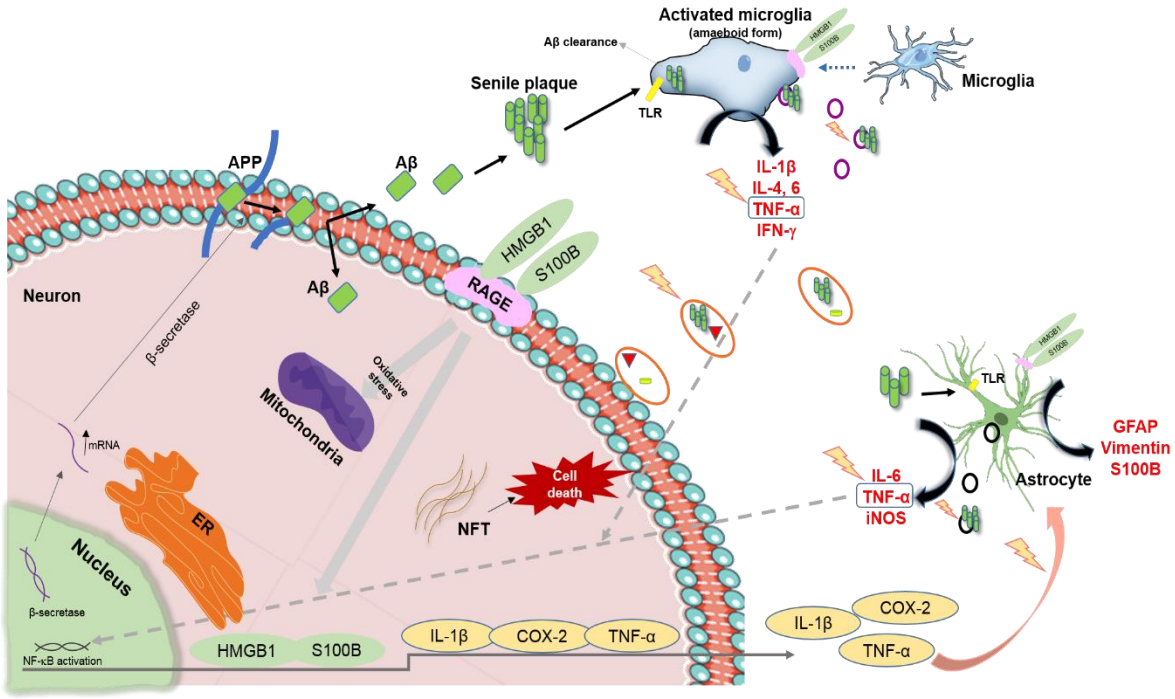

Figure I.4-Schematic representation of the main pathological pathways occurring in Alzheimer's disease. Neuroinflammation has been described for many years as one of the central features involved in Alzheimer's disease (AD) pathogenesis. The amyloid-beta (Aβ) peptide is generated in neurons by β-secretase proteolysis and further senile plaques are formed by Aβ aggregation and sensed by membrane receptors – toll-like receptors (TLRs) – present in microglia and astrocytes. Microglia has a crucial role in inflammation resolution being involved in Aβ clearance. Once activated, microglia change their morphology to an amoeboid shape and become reactive. Inflammatory mediators, namely pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-4, IL-6, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), are involved in neuroinflammatory processes. Reactive astrocytes are also important inflammatory mediators by the release of such pro-inflammatory cytokines to the extracellular space. Moreover, TNF-α activates the nuclear factor kappa B (NF-κB) signaling pathway leading to the upregulation of pro-inflammatory mediators, which in turn activate microglia and astrocytes. β-secretase mRNA expression is also upregulated by pro-inflammatory cytokines. Exosomes delivered by neurons (orange circles), microglia (purple circles) and astrocytes (black circles) may contain Aβ peptides and soluble factors (e.g. pro-inflammatory cytokines), recognized to have a pathological role. High-Mobility Group Box 1 (HMGB1) and S100 calcium binding protein B (S100B) alarmins, released by activated cells, trigger inflammatory pathways by activating the receptor of advanced glycation end product (RAGE) or TLRs on the surface of neurons, microglia and astrocytes, contributing to the neuropathological process. Intracellularly accumulations of hyperphosphorylated tau in neurofibrillary tangles (NFTs) along with endoplasmic reticulum (ER) and mitochondria stress-related are involved as well in AD impairment that culminates with neuronal death.

5. MicroRNA: the emerging roles in Alzheimer's disease

Among proteins and lipids, EVs contain additional nucleic acids, namely RNA species, in their cargo. Among RNA species, miRNAs are able to modify cellular functions and gene expression in the recipient cells (Lafourcade et al., 2016).

MiRNAs, a newly described class containing approximately 22 nucleotide of long non-coding RNAs, act as regulators of gene expression in eukaryotes. Generally, they act as post-transcriptional repressors and may be involved in epigenetic events promoting gene silencing (Breving and Esquela-Kerscher, 2010). MiRNAs can not only mediate gene silencing, but can also promote translational

activation in a cell cycle dependent manner, in which gene activation is only observed in quiescent or arrested cell populations. Cell repression is observed during periods of S/G2 cell growth (Breving and Esquela-Kerscher, 2010).

Biogenesis of miRNAs happens across the nucleus and the cytoplasm by several sequential processing steps by RNase complexes. MiRNAs genes are transcribed by the RNA polymerase II producing pri-miRNA. Pri-miRNA can be processed by two different pathways: canonical and non-canonical pathway (Graves and Zeng, 2012). In the canonical pathway, a pri-miRNA is cleaved by a ribonuclease Drosha liberating a pre-miRNA of approximately 60-70 nucleotides in the nucleus (or hairpin structure precursor). Subsequently, the pre-miRNA is exported to the cytoplasm by Exportin 5 (Exp5) upon guanosine triphosphate (GTP) hydrolysis to guanosine diphosphate (GDP) changing induces Exp5. The pre-miRNA is cleaved by RNase III Dicer, producing a miRNA/miRNA* duplex of approximately 22 base pairs. At the end, an Argonaut protein (Argo), contained in a heterogeneous complex termed RNA-induced silencing complex (RISC), binds the duplex and incorporates the mature single-stranded mi-RNA into the Argo/RNA complex, whereas the other strand mi-RNA* is degraded (Wahid et al., 2010). In the non-canonical pathway, miRNAs do not require all the sequential protein steps (Graves and Zeng, 2012). Non-canonical miRNA biogenesis differs from canonical miRNA biogenesis in that pre-miRNAs are generated by mRNA splicing machinery, dispensing the requirement of Drosha-mediated digestion in the nucleus. Afterwards, the pre-miRNA is exported similarly to the cytoplasm and processed as described in the canonical pathway (Li and Rana, 2014).

MiRNAs are recognized as key regulators of several biological functions such as neurite outgrowth, dendritic spine morphology, neuronal differentiation and synaptic plasticity (Breving and Esquela-Kerscher, 2010). However, altered expression of miRNAs is increasingly known as a feature of many diseases status, including neurodegeneration (Goodall et al., 2013). Recent studies have demonstrated their particular importance in synapses, where EVs carrying miRNAs play a major role in both physiological and pathological conditions (Olde Loohuis et al., 2012). Therefore, profiles of deregulated miRNAs may be used as non-invasive potential biomarkers in neurodegenerative disorders (LM et al., 2012; Leidinger et al., 2013), along with neuro-imaging parameters, individual or proteomic profiles in the CSF, blood, plasma or serum (Lugli et al., 2015). Furthermore, exosomal miRNAs can exist stably in blood, urine, CSF and other body fluids reflecting their tissue or cell of origin (Zhang et al., 2015). There are different methods to measure miRNAs in samples, including the use of microarrays and quantitative polymerase chain reaction (qPCR) (Femminella et al., 2015).

Recently, miRNAs have been implicated in the molecular pathogenesis of AD acting as mediators of the inflammatory response, whereby are referred as inflamma-miRs and found associated to both genetic and sporadic disease etiology (Olivieri et al., 2013; Hu et al., 2016).

5.1. MicroRNA–21

MiR-21 was shown to be involved in many biological functions, including development, and in diseases like cancer, inflammation, cardiovascular disorders and, more recently, in Spinal cord injury (SCI) (Bhalala et al., 2013). It has been described as the most commonly over-expressed miRNA in

cancer (Ribas et al., 2012), being considered an onco-miRNA. However, its role in the CNS is still unknown (Fu et al., 2017).

MiR–21 is located on chromosome 17q23.2 within the intronic region of the TMEM49 gene. Despite of being both transcribed in the same direction, pri-miR-21 has its own promoter and poly(A) trail (Kumarswamy et al., 2011).

Overstimulation of NMDA receptors has been implicated in many neurodegenerative diseases and long considered a mechanism of neurological injury (Lipton and Rosenberg, 1994). Yelamanchili and colleagues demonstrated that stimulation of NMDA receptors in human and monkey donors lead to a significant upregulation of miR-21, without increase of cell death. Moreover, using motor neurons transfected with lentivirus expressing miR-21 and electrophysiological stimulation, they have seen increased outward K^+ currents in neurons transfected with miR-21, when compared to neurons, suggesting its pathogenic role in neurodegenerative diseases (Yelamanchili et al., 2010).

In a study involving SCI condition, upregulation of miR-21 was found to be implicated in astrocytes hypertrophy, whereas its inhibition increased axon density within lesion site. These evidences indicate a role for miR-21 in astrocyte hypertrophy and glial scar progression, and that its modulation may have potential therapeutic benefits by manipulating gliosis and enhancing functional outcome (Bhalala et al., 2013).

MiR-21 can be also upregulated by LPS stimulus in order to resolve inflammation. It appears that it promotes IL-10 expression by inhibiting PDCD4 acting as an IL-10 inhibitor. Furthermore, it contributes for apoptosis attenuation because it suppresses several pro-apoptotic genes (Ponomarev et al., 2013). Such effect was observed in neurons after traumatic brain injury (Han et al., 2014). However, when in EVs, miR-21 was shown to be able to trigger neurotoxicity via TLR signaling (Yelamanchili et al., 2015)

Not much is known about the involvement of miR-21 in AD, although Schonrock and colleagues observed a moderate decrease of miR-21 upon exposure to Aβ in an *in vitro* model. To see if this also occurred in an *in vivo* model, they used an APP23 mice model at different ages. MiR-21 maintained its significant downregulation with ageing suggesting that Aβ acts as a regulator in both *in vitro* and *in vivo* models (Schonrock et al., 2010). Indeed, back in 2013, Petra and colleagues showed a time-dependent miR-21 downregulation in murine primary hippocampal cell cultures after neuronal Aβ treatment, corroborating other previous results (Leidinger et al., 2013).

5.2. MicroRNA-124

MiR-124 is the most abundant miRNA in the CNS and preferential expressed in brain, retina and spinal cord neurons. There are three different miR-124 genes, miR-124-1, miR-124-2 and miR-124-3 located on three different chromosomes either in human or mouse genome (Lagos-Quintana et al., 2002; Yu et al., 2008).

During development, miR-124 expression increases in parallel with neuronal maturation. Its abundance in the subventricular zone is determinant in neuronal fate, being Sox9 its physiological target, which is responsible for such effect (Sun et al., 2015). Its role in neurite outgrowth is also well known (Yu et al. 2008).

MiR-124 has been implicated in many neurodegenerative diseases, including AD. Previous results showed an inverse relationship between miR-124 and BACE1. In other words, while miR-124 is down-regulated in AD neurons, BACE1 is upregulated, thus increasing Aβ peptide levels. These results were shown when transfected miR-124 inhibitor in a disease cell model led to a remarkable increase in BACE1 expression (Fang et al., 2012a). On the other hand, in microglia and macrophages, miR-124 was demonstrated to be important for the development of myeloid cells by targeting CEBPα and PU.1, known as two crucial transcriptional factors (Ponomarev et al., 2013). In the same study by Ponomarev, it was found that miR-124 also contributed to a more "calming" microglia phenotype by targeting the CEBPα/PU.1 signaling pathway. In agreement the surveiling, also called "resting" microglia showed low levels of CEBPα, while when activated there was CEBPα upregulation (Ponomarev et al., 2013). Most interesting, miR-124 was demonstrated to control the choice between neuronal and astrocyte differentiation. In other words and in this context, miR-124 represses the expression of a critical epigenetic factor lysine methyltransferase Ezh2 that promotes neuronal and counters astrocyte-specific differentiation route (Neo et al., 2014).

5.3. MicroRNA-155

MiR-155 is considered a multifunctional miRNA being involved in several biological processes, including hematopoiesis, inflammation and immunity (Faraoni et al., 2009). Due to such multifunction ability, it is reasonable to consider that many other functions will be revealed in the near future. Its expression is more elevated in the adult brain than during development (Leong et al., 2014).

Regarding inflammation, miR-155 expression is considered to constitute one of the primary immune cells response to different inflammatory mediators. LPS, IFN-β, TNF-α or polyriboinosinic-polyribocytidylic acid [poly(I:C)] have been pointed as being capable of inducing miR-155 upregulation. Many of its targets identified so far are pro- and anti-inflammatory proteins, such as Fas-associated death protein, IκB kinase-ε, inositol 5-phosphatase 1 and the suppressor of cytokine signaling-1 (SOCS-1). SOCS-1 acts by directly targeting JAK and, consequently, by inhibiting STAT signaling pathway as a "classical" negative feedback effect. Moreover, miR-155 plays a pro-inflammatory role during microglia activation being implicated in chronic inflammation and contributing to AD pathogenesis (Cardoso et al., 2012). It is implicated in the downregulation of M2 associated genes and recognized to downregulate the IL-13 receptor and to target SMAD2 involved in the anti-inflammatory signal transduction in TGF-β pathway associated with the M2 phenotype (Ponomarev et al., 2013). MiR-155 upregulation was observed in the 3xTg AD mice and in Aβ-activated microglia and astrocytes (Guedes et al., 2014), but was shown to have no changes in astrocytes in the context of chronic active multiple sclerosis cases (Rao et al., 2016a). Interesting, in an *in vivo* model of ALS, miR-155 was demonstrated to be significantly upregulated before disease onset and in the absence of other common pro-inflammatory specific markers. Such evidences may suggest that its presence, instead of being associated with a neuroinflammatory status, may have an anti-inflammatory role to retain the disease progression in the presymptomatic stage (Cunha et al., 2017)

When it comes to the potential therapeutic roles, the application of pro-inflammatory miRNAs like miR-155 may reveal useful upon insufficient activation of macrophages in order to promote the clearance of amyloid plaques (Ponomarev et al., 2013).

5.4. MicroRNA-146a

In AD, a range of deregulated miRNAs have been described, but miR-146a stands out as having a central role in multiple pathways in AD and to be elevated in diverse biological samples early in the disease. Despite its well-known role in the innate immune response, miR-146a is abundant in both mouse and human brain, being expressed in microglia, astrocytes and, more importantly, in neurons (Gang Wang et al., 2016). Moreover, miR-146a levels were found to progressively increase with the disease severity and to co-localize in brain regions showing inflammatory neuropathology (Lukiw et al., 2013).

MiR-146a is expressed in human brain in astrocytes, as state before, in which are indicated as key targets in the regulation of this miRNA in response to inflammatory molecules. Indeed, regarding AD, upregulation of miR-146a has been detected in AD brains suggesting a role in governing astrocytes activation and function. Additionally, upon exposure to IL-1β, IL-6, TNF-α or HMGB1, astrocytes exhibit upregulation of miRNA-146a (Iyer et al., 2012). Recently, it was shown that miR-146a produced by astrocytes contributes to motor neuron loss, at least in spinal muscular atrophy (Sison et al., 2017). Moreover, in a study undertaken by Alexander and colleagues, they've seen that both miRNA-155 and miRNA-146a were released via exosomes from dendritic cells. Interestingly, they've also seen that injections of miR-146a-containing exosomes in mice exposed to LPS inhibited their inflammatory response, whereas miRNA-155 promoted inflammation (Alexander et al., 2015).

5.5. MicroRNA-125b

MiR-125b is the human orthologue of *lin-4*, one of the first miRNA identified in *C. elegans*, that was shown to be important for post-embryonic proliferation and differentiation mechanism in the warm (Abbott, 2011). MiR-125b belongs to the miRNA-125 family, composed by miR-125a, miR-125b and mirR-125b-2, expressed by distinct genes (Banzhaf-Strathmann and Edbauer, 2014). Some studies revealed its downregulation in colorectal, breast, gastric and non-small cell lung cancers, as well as in glioblastomas (Xie et al., 2013).

Physiologically, miR-125b was shown to promote the differentiation of SH-SY5Y cell line (Le et al., 2009). These authors showed that upregulated genes by ectopic expression of miR-125b revealed to be related with important biological processes, such as nervous system development, neurite outgrowth, cell adhesion, cell morphology, mobility and cytoskeleton organization. On the other hand, downregulated miR-125b determined severe developmental defects and elevate p53 level and apoptosis (Le et al., 2009).

MiR–125b is described as one of the most abundant miRNAs in the brain, especially in mature neurons (Le et al., 2009), being significantly elevated in AD patients. Curiously, Rao et al, have shown that human astrocytes from deeper grey matter do not express miR-125b (Rao et al., 2016b). Some studies have revealed that miR-125b causes downregulation of the essential synaptic glycoprotein synapsin-2, an important protein for the activity of synaptic vesicles and for synaptic transmitter trafficking in neuronal circuit. Concerning Aβ and tau, miR-125b is predicted to be a regulator for APP processing, as well as Aβ degradation genes and, overexpression of miR-125b was shown to

significantly increase tau hyperphosphorylation (Li et al., 2017). In a previous study using lentivirus vectors expressing miR-125 precursor, it was also shown that miR-125 overexpression increased tau phosphorylation and reduced cell viability by 20%, when compared with control, further suggesting miR-125 potential neurotoxic effects (Banzhaf-Strathmann et al., 2014). These authors also observed that miR-125 overexpression impaired learning and memory in an *in vivo* mouse model of AD.

6. *In vitro* cells models in Alzheimer's disease pathogenesis study

Cell culture has been for many decades the back-bone of basic biomedical research, wherein, both normal and pathological cellular processes have been studied. The majority of the cellular lines used in biomedical research carry genetics and epigenetics features of accommodation to tissue culture, being either derived from malignant tissues or genetically modified to grow infinitely (Grimm, 2004).

In order to study the underlying pathogenesis mechanisms of AD, once it is very difficult to work with patient brain cells, rodent primary cultures of neural cell types and cell lines have been the resource model for the majority of studies performed up to now. Cell lines usually derive from cancer (e.g. neuroblastoma or glioblastoma). The major problem of these models in many fields of neuroscience, especially in AD, is the lack of resemblance with functionally mature neurons that express human proteins. Despite of this, immortalized cell lines (Agholme et al., 2010) and transfection with genes associated with AD (PSEN1, PSEN2 or APP) have been largely used. Recently the possibility to collect skin cells from patients with AD and the possibility to create *in vitro* models that recapitulate AD was an important advance (MN et al., 2013). Indeed, induced pluripotent stem cells (iPSCs) derived from patients fibroblasts were shown to better mimic AD and to be important in disease modeling and cell-replacement therapy (Yang et al., 2016).

In conclusion, human cell lines, primary cultures, animal models and, now, iPSCs may work in a complementary way to assess the complex dysfunctional mechanisms that have been described as potentially implicated in such disorder.

6.1. Human neuroblastoma SH-SY5Y cell line

SH-SY5Y cell line is a subclone of the SK-N-SH cell line obtained from a bone marrow biopsy of a neuroblastoma patient. It is an immortalized and proliferative cell line and due to their ability and potential to differentiate into a neuronal phenotype it is most used in *in vitro* studies of neurodegenerative diseases, like AD and PD (Forster et al., 2016).

Undifferentiated SH-SY5Y cells lack many of the features that define neurons, namely alterations in neuronal morphology, inhibited cell division and changes in the expression of neuron-specific markers. Moreover, this cell line also rarely expresses neuron-specific proteins at levels comparable to mature neurons. However, SH-SY5Y cells show some advantages as the possibility to be differentiated into several different phenotypes depending on the addition of specific factors (Agholme et al., 2010). The most common method for differentiation uses retinoic acid (RA), which induces morphological changes, neurite growth and expression of neuronal markers (Xun et al., 2012). Consequently, these differentiated cells have synaptic structures, functional axonal vesicles transport and express neurospecific proteins, including nuclear protein NeuN, neuron specific class III β-tubulin and synaptic protein Sv2 (L et al., 2010).

SH-SY5Y cells expressing APP_{695} Swedish mutation (SH-SY5Y APP_{Swe}) were obtained by transfecting of the pIREShyg vector encoding human APP_{Swe} cDNA in a study conducted by Belyaev in order to understand the underlying mechanism of APP amyloidogenic processing (Belyaev et al., 2010). This study revealed that only APP_{695} isoform was able to upregulate AICD levels, as well as metallopeptidase neprilysin (NEP) gene expression, involved in Aβ degradation, when compared with other alternative APP spliced isoforms (APP_{751} and APP_{770}). Moreover, SH-SY5Y APP_{Swe} showed increased levels of Aβ and NEP expression when compared with wild-type APP_{695} expressing cells.

6.2. Human CHME3 microglial cell line

CHME3 microglial cell line was firstly obtained by transfecting with simian virus 40 large T antigen of a human primary microglia from 8-10 weeks embryos, retaining its properties. Such microglia revealed a homogeneous cell population that can be grown indefinitely, and have been used as an *in vitro* model system in several studies, such as regulation of gene expression (Janabi et al., 1995), effect of immunomodulatory substances (Hjorth et al., 2010) or to model diseases (Haedicke et al., 2009).

Hjorth and colleagues evaluated the response of CHME3 cell line to the exposure of $Aβ_{1-42}$ as an *in vitro* cell model of AD. In such study, CHME3 cells exposed to $Aβ_{1-42}$ in combination with IL-1β and IFN-γ revealed reduced brain derived neurotrophic factor (BDNF) secretion and increased IL-6 release. Regarding phagocytosis of $Aβ_{1-42}$, CHME3 cells were able to uptake the oligomer and, interestingly, it co-localized with lysosomes (Hjorth et al., 2010). These results, among others described in literature, suggest that Aβ (the major component of senile plaques) is involved in microglia activation in AD.

6.3. Human Induced Pluripotent Stem Cells (hiPSCs)

Back in 2006, Yamanaka and his team has taken skin cells from adult mice and infected them with a virus designed to introduce 24 carefully chosen genes, resulting in the transformation of the cells. These cells started to behave like embryonic stem cells (ESC), with the ability to differentiate in practically any cell type. Afterwards, Yamanaka narrowed down the genes to just four that were needed to wind back the development clock.

The identification of genes responsible for reprogramming cells were published later on, being called as Yamanaka factors (i.e. Oct4, KLF4, Sox2, and c-Myc), improving the method for reprogramming cells. With their huge potential, labs started to adopt iPSCs becoming an important tool not only for modeling and investigating human diseases, but also for screening of new drugs (Sproul, 2015).

6.3.1. Cellular reprogramming of somatic cells and generation of iPSCs

A fundamental question in the regeneration field is to find cells capable of maintaining the ability to differentiate into the three germ layers: endoderm, ectoderm and mesoderm. Embryonic stem cells are cells with the "pluripotency" capability, however due to ethical questions alternative strategies were required (Mungenast et al., 2016). So, the turning point was achieved with the discovery that somatic cells (fibroblast, blood, etc) could be reprogrammed to a pluripotent state. Since the original demonstration that fibroblast could be reprogrammed to become iPSCs, by Yamanaka, several methods have been generated to create iPSCs (Malik and Rao, 2013). These methods include for instance the

use of vectors with Cre-Lox recombination or reprogramming by nonintegrating viruses (Malik and Rao, 2013).

6.3.2. iPSCs as a novel and promisor *in vitro* Alzheimer's' disease model

The use of post-mortem tissues and transgenic animal models has been crucial to elucidate the pathogenesis of several neurodegenerative diseases. However, these kind of models lack in a way that post-mortem tissues are not always available and often represent the end stage of the disease while any animal cell line does not fully recreate true AD (Goldstein et al., 2015).

The discovery of iPSCs and the conversion of somatic cells into specific brain cell types opened a new window to explore implications of a specific genotype authentically expressed in a cell type specific context (Yang et al., 2016). In AD, human iPSCs (hiPSCs) derived from FAD and SAD patients' somatic cells contain patient-specific pathogenic background, an advantage for AD modeling and a point break between animal models and clinical testing. Since the discovery of the potential of such cells, several research groups were able to demonstrate that hiPSCs recapitulate some pathological features of AD, when used in *in vitro* systems (Yang et al., 2016).

For instance, iPSCs from AD patients with PSEN 1, PSEN 2 and APP familial mutations have already been generated (Yagi et al. 2011; Israel et al. 2012). Due to the capacity to generate any cell type, it is now possible to generate neural progenitor cells (NPCs), and consequently obtain neurons, astrocytes and oligodendrocytes (Zhou et al., 2016).

Neurons differentiated from SAD- and FAD-iPSCs were shown to resemble pathologically affected cells *in vivo* and, importantly, to express key disease hallmarks (Israel et al., 2012; Mohamet et al., 2014). The majority of AD iPSCs models so far have used monolayer cultures of mixed neurons population and they have already given important insight into AD pathogenesis. However, iPSCs, as already mentioned, have the potential to generate specific cell types that can be cultured alone or in combination with neurons thus creating much richer and reflective models of human disease (Sproul, 2015). The loss of normal astrocyte function has been pointed out as a primary contribution to neurodegeneration, highlighting the role of astrocytes in the neurodegenerative field. Therefore, generation of robust and homogeneous astrocytes populations from iPSCs of patients would be a valuable tool to reveal astroglial contribution to neurodegenerative diseases (Jones et al., 2017).

Although animal models have provided essential insights into the role of astrocytes in disease context, human astrocytes display a more complex structure than rodents in terms of size and functional processes (Tyzack et al., 2016). The first human astrocytes were cultured from fetal and post-mortem tissues but we have to bear in mind that biopsies represent the end stage of the disease and control tissue is often inaccessible due to ethical reasons and potential health risks (Chandrasekaran et al., 2016). In a recent report, astrocytes from hiPSCs derived from patients with FAD and SAD and health control were generated (Jones et al., 2017). It was found that AD patients' astrocytes express the same canonical markers of mature healthy astrocytes, however their morphological appearance and cellular phenotype is significantly distorted (Jones et al., 2017). In the same study, authors did not observe any differences in early neuronal commitment in AD-derived neurons compared with healthy controls. These findings indicate the cell autonomous pathological potential of astrocytes while suggests that cellular pathology does not come from neural progenitors. Moreover, astrocytic atrophy may act as a plausible

mechanism for early cognitive impairment, providing a potential novel therapeutic target for AD intervention (Jones et al., 2017). In another study, aberrant astrocytes differentiated from HD-iPSCs showed a vacuolation phenotype, a phenomenon already documented in primary lymphocytes also from HD patients, suggesting a specific HD phenotype (Juopperi et al., 2012).

To further confirm whether the disease is due to genetic mutations or other autonomous factors, researchers have developed a new strategy, the use of isogenic iPSCs cell lines. Here, the mutation of interest is corrected in a patient-derived cell line through DNA techniques repair. The advantage of using isogenic cells is that only the disease-associated differences are studied, while if disease pathogenesis is mutation-dependent it is expected that isogenic cells will behave as a control line (Mungenast et al., 2016).

The development of efficient and reliable ways to make genetic modifications has been, for many years, the long-standing goal for biomedical researchers. The discovery of a tool based on a bacterial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein-9 nuclease (Cas9) has generated considerable excitement (Reis et al., 2014). Based on this novel and promising tool it is possible to knockout specific genes or repair gene mutations and further expands the utility of hiPSCs (Chen et al., 2015). Regarding this, isogenic controls derived from AD-iPSCs, in which the mutation has been restored to wild type (wt), are the most appropriated control cells to discover and elucidate more accurate features and mechanism of AD pathogenesis (Yang et al., 2016).

7. Aims

The global aim of the thesis is to investigate how cell-to-cell communication may be deregulated in AD using *in vitro* models. First, we intent to identify the human SH-SY5Y and SH-SY5Y APP_{Swe} exosomes cargo, and how they influence the activation and dysfunction of the recipient human microglial cells and their phenotype/inflammatory response. Secondly, we aim to characterize hiPSCs-derived astrocytes from AD patients in what concerns to exosome secretion, inflammatory microRNAs and inflammatory response.

Therefore, the specific aims are to:

1. Evaluate whether SH-SY5Y cell line expressing APP_{Swe} and respective exosomes have a different microRNAs and inflammatory cytokines profile.
2. Identify if CHME3 microglial cell line (recipient cells) incorporate and react differently to exosomes from SH-SY5Y APP_{Swe} (donor cells).
3. Assess whether hiPSCs-derived astrocytes from AD patients expressing PSEN1ΔE9 mutation show a distinct reactive phenotype when compared to matched controls.

II. Materials and Methods

1. Materials

1.1. Supplements and Chemicals

Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 w/o glutamine, N2 supplement 100x, Non-essential amino acid cell (NEAA), GlutaMAX 100x, Pen/Strep, Heparin, LysoTracker™ Red DNA-99 were purchased from Thermo Fisher Scientific (Waltham, MA, USA); fetal bovine serum (FBS), antibiotic/antimycotic (AB/AM), L-glutamine (L-glu) were purchased from Biochrom AG (Berlin, Germany); Basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), Ciliary Neurotrophic Factor (CNTF) and Bone Morphogenic Protein 4 (BMP4) were obtained from PeproTech (London, UK); LDN193189 inhibitor from Selleckchem (Munich, Germany); accutase from STEMCELL™ Technologies (Grenoble, France); SB431542 inhibitor; trypsin-EDTA solution (1X), trypsin-EDTA solution (10X), Hoechst 33258 dye, bovine serum albumin (BSA) and PKH67 Fluorescent Cell Linker Kit were from Sigma-Aldrich (St. Louis, MO, USA); Triton X-100 was obtained from Roche Diagnostics (Mannhein, Germany); paraformaldehyde (PFA) and Guava Nexin® Reagent were purchased from Merck Millipore (Darmstadt, Germany); TRIzol® reagent and primers for HMGB1, S100B, RAGE, TNF-α, IL-10, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Invitrogen Corporation™ (Carlsbad, CA, USA); miRCURY™ RNA Isolation Kit and miRCURY LNA™ Universal RT microRNA PCR (Universal cDNA Synthesis Kit II, ExiLENT SYBR® Green master mix and UniSpike in) were purchased from Exiqon (Vedbaek, Denmark), as well as the PCR primer mixes for miR-124, miR-21, miR-146a, miR-155, miR-125b and U6; SYBR® Green RT-PCR Reagents Kit was obtained from Applied Biosystems™ (Foster City, CA, USA). All the other common chemicals were purchased either from Sigma-Aldrich or Merck.

1.2. Equipment

To maintain a stable environment to optimal cell growth (37°C and 5% CO_2), cell cultures were kept in a HERAcell™ 150 incubator (Thermo Fisher Scientific, Waltham, MA, USA) and all the experimental work was performed in sterile conditions using a Holten Lamin Air HVR 2460 (Allerod, Denmark).

For exosome isolation, we used a Beckman Optima™ L-100 XP ultracentrifuge, with a type 90 Ti rotor (fixed angle) and centrifuge bottles of polycarbonate, from Beckman Coulter, Inc. (Fullerton, CA, USA). Fluorescence microscope (model AxioScope.A1) coupled with an AxioCam HR camera was purchased from Carl Zeiss, Inc. (North America). For protein quantification we used a microplate reader (PR 2100 Microplate Reader). Western Blot images were obtained from ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). For the viability assay, we used The Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore, Darmstadt, Germany). Total RNA was quantified using NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For synthesis of cDNA it was used Biometra® Tpersonal Thermocycler (Göttingen, Germany). For determination of mRNA and miRNA expression, by quantitative Real Time PCR (RT-PCR), it was used the QuantStudio™ 7 Flex Real-Time PCR System, from Applied Biosystems™ (Foster City, CA, USA).

Eppendorf 580R (Eppendorf, Hamburg, Germany) and Sigma 3K30 centrifuges were used for different experimental procedures.

1.3. Antibodies

The primary antibodies used in this work were rabbit Microtubule-associated protein 1A/1B-light chain 3 (LC3) (1:200), purchased from Cell Signaling Technology® (Danvers, MA, USA), Glial Glutamate Transporter (GLT-1) (1:180) and S100B (1:200), obtained from Abcam (Cambridge, UK), and GFAP (1:100), from Novocastra. The secondary antibodies used were Alexa Fluor® 488 and Alexa Fluor® 594 goat anti-rabbit (1:1000) and Alexa Alexa Fluor® 488 goat anti-mouse (1:1000), obtained from Invitrogen Corporation™ (Carlsbad, CA, USA).

2. Methods

2.1. Cell lines and treatment

2.1.1. Human SH-SY5Y and SH-SY5Y APP Swedish neuroblastoma cell line

Human neuroblastoma SH-SY5Y and SH-SY5Y stably expressing APP695 Swedish mutation (SH-SY5Y APP_{Swe}) cells were a gift from Professor Anthony Turner. SH-SY5Y cells were used as controls. Both cell types were routinely cultured in T75 in DMEM (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 2% AB/AM. All cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37ºC. Medium was changed every 2 to 3 days. For cell characterization, cells were seeded on 12-well plates coated with poly-D-lysine (100 µg/mL) and laminin (4 µg/mL) at a final concentration of $5x10^4$ cells per well.

After 24h proliferation (day 1), differentiation was induced by adding retinoic acid (RA) (Sigma-Aldrich) at a final concentration of 10 µM in culture medium and maintaining cells for 7 days (day 8). RA-containing culture medium was changed every 2 days. At day 8, medium was changed to DMEM basal medium (FBS free) and at day 9 cells were collected for RNA extraction, immunostaining and respective exosomes isolated from culture medium for analysis. In parallel, isolated exosomes were labelled with PKH67 fluorescent probe as described in **section 2.2.2** (figure II.1), prior to incubation on CHME3 microglial cells.

2.1.2. Human CHME3 microglia cell line

Human CHME3 microglial cells, a gift from Professor Marc Tardieu, were routinely cultured in T75 in DMEM (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 2% AB/AM (Sigma-Aldrich, St. Louis, MO, USA) and 1% L-glu (Sigma-Aldrich) in a humidified atmosphere containing 5% CO_2 at 37°C. Medium was changed every 2 to 3 days. For cell characterization, cells were seeded on 12-well non-coated plates at a final concentration of $5x10^4$ cells per well and allowed to adhere and stabilize for 24h. Then, to determine the different effects of exosomes on microglia, cells were incubated with DMEM, either alone (control) or containing exosomes from SH-SY5Y or SH-SY5Y APP_{Swe}. Microglia response was evaluated upon incubation with exosomes for 24 or following another 24h in free-exosome media (total period 48h) (Figure II.1), in order to evaluate the response of healthy microglia to a short exposure to exosomes (24 h) and later response after another 24h of recovery (48 h). At the end of each time point, exosomes were collected to evaluate inflammatory

related markers, while coverslips attached cells were either (i) fixed for 20 min with freshly prepared 4% (w/v) PFA in phosphate buffered saline (PBS), for immunocytochemical studies; or (ii) covered with TRIzol, for RNA isolation.

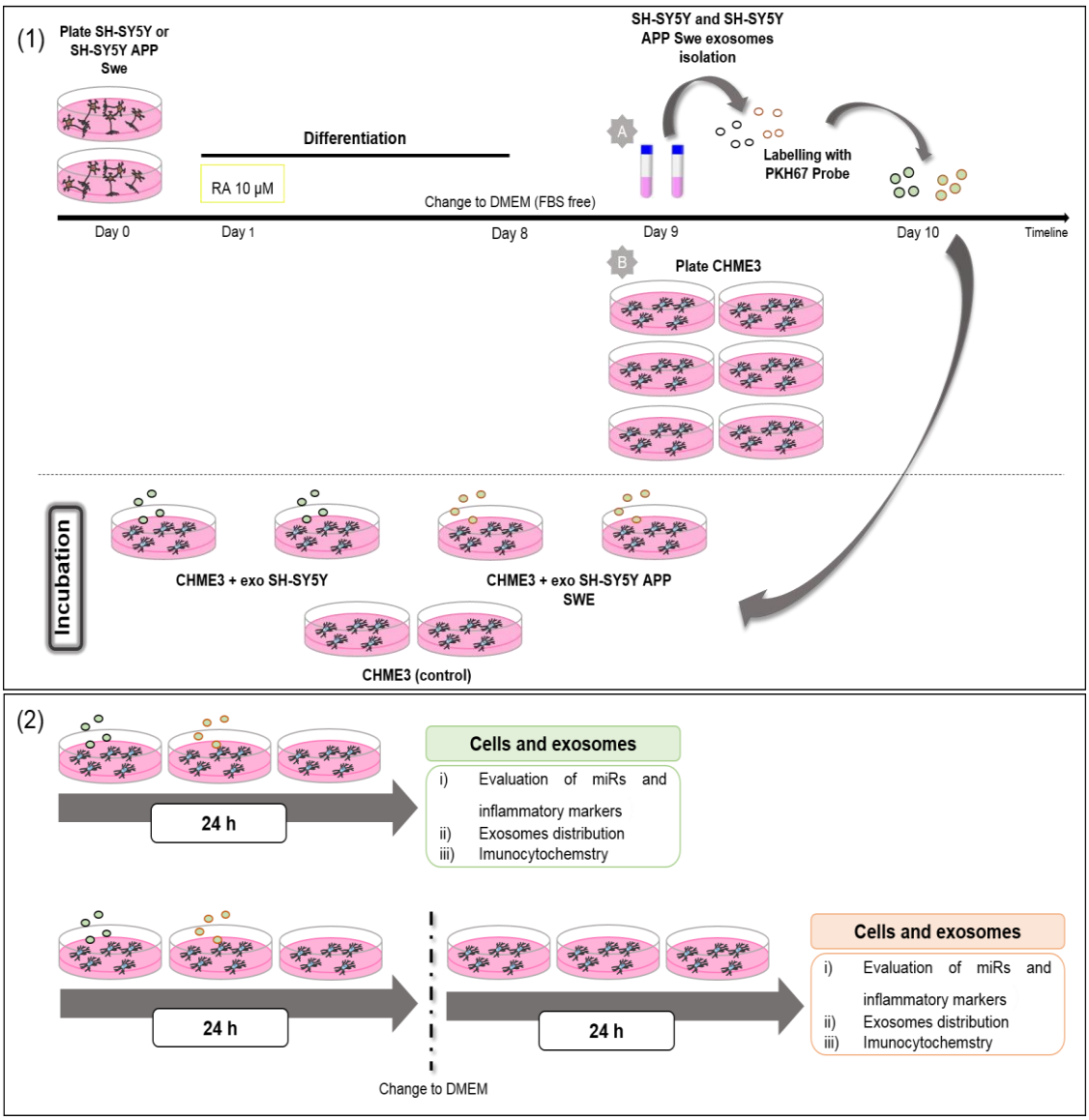

Figure II.1-Schematic representation of the experimental model for human neuroblastoma cell lines, isolation of exosomes and incubation with human microglia. (1) Neuronal and microglial cultures, and isolation of exosomes. SH-SY5Y and SH-SY5Y APP$_{Swe}$ cells were differentiated for 7 days in vitro (DIV). At day 9, cells were collected to evaluate inflammatory-associated genes and miRNAs by RT-PCR. Exosomes were isolated from extracellular media (FBS free) by differential ultracentrifugation followed by labelling with the PKH67 probe. CHME3 cells were maintained in culture for 24 h (from day 9 to day 10) and then incubated with SH-SY5Y and SH-SY5Y APP$_{Swe}$-derived exosomes **(2) Incubation of the isolated exosomes with microglia and evaluation of cell activation and dysfunction.** Neuroblastoma-derived exosomes were incubated with microglia for 24 h. In one set of experiments CHME3 cells were evaluated at the end of the 24 h incubation period; in another set of experiments previous cells were allowed to recover in exosome-free media and evaluated at the end of an additional period of 24 h. At each time point, cells and exosomes of each set were evaluated for (i) expression of inflammatory miRNAs and mRNA markers by RT-PCR; (ii) intracellular distribution of exosomes; (iii) activation of lysosomal function, autophagy and Aβ-uptake, following Lysotracker®, and immunocytochemical staining of LC3 and Aβ, respectively.

2.1.3. Astrocytes derived from hiPSCs

Astrocytes were differentiated from spheres derived from hiPSCs (Figure II.2) prepared at Jari Koistinaho lab as indicated in Figure II.2. Briefly, iPSCs derived from somatic cells are seeded in matrigel covered plates and kept in culture in Neural differentiation medium (NDM) supplied with sodium butyrate and LDN. iPSCs colonies are formed within the first 11 days and after that iPSCs rosettes start to appear and confirm the presence of neuroepithelial differentiation. From day 14 until day 18, iPSCs rosettes are maintained in NDM supplied with bFGF. At day 18 rosettes are transferred to Ultra-low attachment (ULA) plates and kept in culture in astrodifferentiation medium supplied with bFGF and EGF. At day 22, spheres are visible and give rise to predominantly neurons in the following month before gliogenesis. After about 5 months, astroglial progenitor cells and astrocytes can be identified by immunodetection of S100B and CD44, thus the spheres are referred to as astrospheres. These astrocytes and glial progenitors can be expanded for additional months as astrospheres or as spheres-reforming monolayers (Krencik and Zhang, 2011).

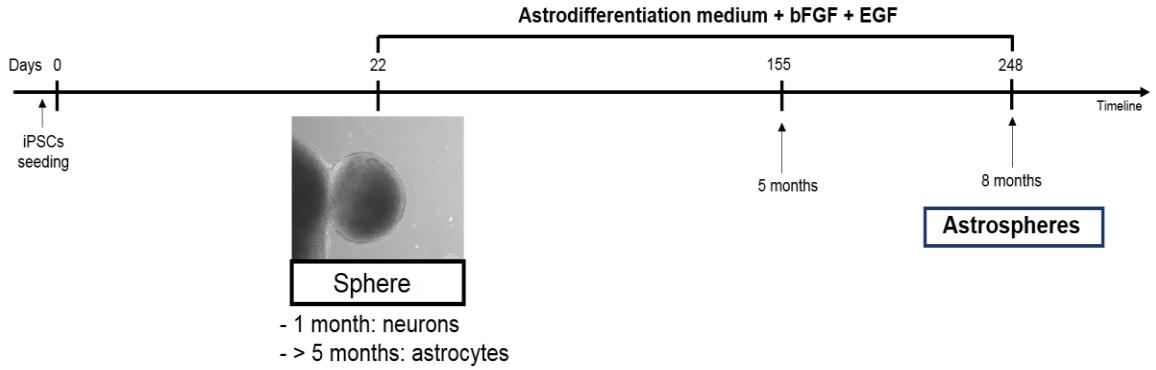

Figure II.2-Representative timeline to differentiate astrospheres from iPSCs. iPSCs are cultured in a matrigel coated flasks from day 0 to day 22 in a Neural differentiation medium (NDM) supplied with different growth factors according to iPSCs differentiation stage. Once spheres are formed, they are transferred to a Ultra-Low adherent (ULA) dish and with an appropriated astrodifferentation medium more than 5 months are required to obtain well differentiated astrocytes.

Astrospheres from cell lines described in Table II.1 were routinely cultured in ULA 10 cm dishes (Corning, NY, USA) in astrodifferentiation medium [DMEM/F12 w/o glutamine (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1% N2 supplement 100x, 1% NEAA, 1% GlutaMAX and 0,5% Pen/Strep (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA)], in a humidified atmosphere containing 5% CO_2 at 37°C, and cut 1 to 2 times per week. Half of the total medium volume was changed every other day by new astrodifferentiation medium supplemented with the growth factors bFGF (10 ng/ml) and EGF (10 ng/ml). To each new experiment, around 50 media spheres from the 6 different cell line referred above, were collected to a 15 ml tube. Spheres were chemically dissociated with accutase (Sigma) for 5 min at 37°C. Afterwards, astrocytes were mechanically dissociated with the use of a micropipette and cells counted. Astrocytes were seeded in 12-well plates coated with Matrigel Matrix (Corning, NY, USA) at a final concentration of $2x10^5$ cells per well in astrodifferentiation medium supplemented with BMP4 (10 ng/ml) and CNTF (10 ng/ml) and kept in culture for 8 days. Medium was changed every 2 days. At day 6, medium was collect for exosomes isolation and replaced by freshly

medium until day 8 where once again medium was collected for exosomes isolation. Isolated exosomes from day 6 and 8 were mixed to increase particle and RNA yield. Cells were collected at day 8 to RNA extraction or immunostaining, as described above.

Table II.1-Genotype description of iPSCs-derived astrocytes.

Cell name	Cell genotype	Respective isogenic cell
Control 8.2 **Control 8.7**	**Two copies of Apo E ε3 allele** (E3/E3) without known risk factors and family history of AD.	
AD 5 1.5	FAD cells obtained from two patients carrying **exon 9 deletion mutation of PSEN1** (PSEN1ΔE9) and free of other known genetic risk factors.	AD 5 isogenic
AD 4 1.6		AD 4 isogenic

.

2.2. Isolation of extracellular vesicles, exosome labeling and characterization

2.2.1. Exosomes isolation

Extracellular vesicles were isolated from media of SH-SY5Y, SH-SY5Y APP_{Swe} and CHME3 in monoculture and following CHME3 incubation with SH-SY5Y or SH-SY5Y APP_{Swe} derived exosomes as indicated in Figure II.3. Briefly, cell supernatant was centrifuged at 1 000 *g* for 10 min, to pellet cell debris. Then, the supernatant was transferred to another tube and centrifuged at 16 000 *g* for 1 hour, to pellet microvesicles. These microvesicles were washed in PBS (16 000 *g* for 1h) and the remaining supernatant was filtered in a 0.22 µm pore filter to remove particles above 200 nm and centrifuged at 100 000 *g* for 2 hours. The exosomes pellet was then resuspended in PBS and centrifuged one last time at 100 000 *g* for 2 hours, in order to wash it. Exosomes were then used for labelling, characterized by Nanoparticle Tracking Analysis or resuspended in 200 µl of lysis buffer from miRCURY™ RNA Isolation Kit for evaluation of miRNAs and mRNA content.

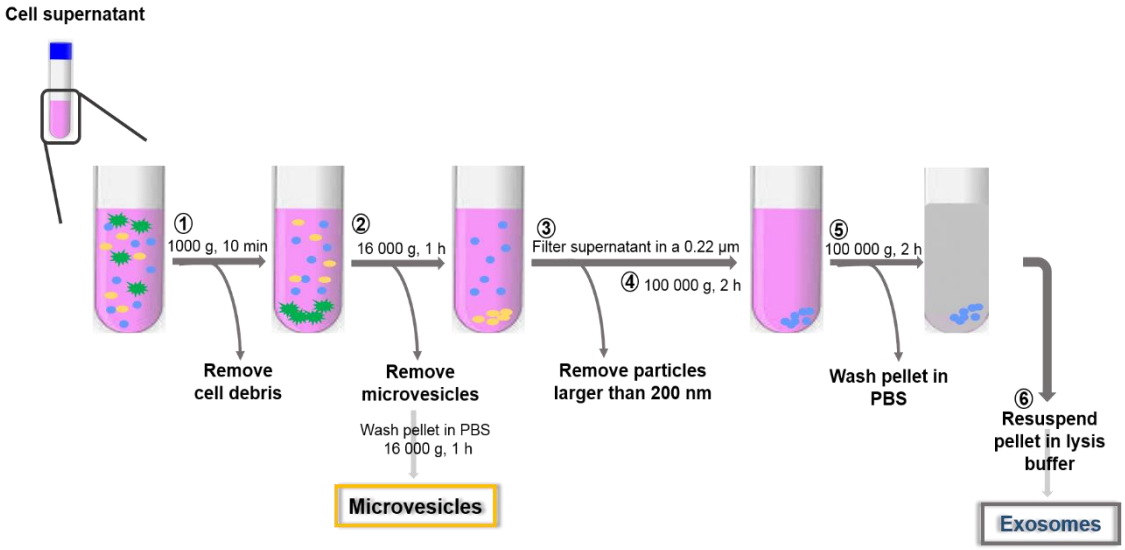

Figure II.3-Schematic procedure for isolation of exosomes from cell extracellular media. (1) Cell supernatants are centrifuged at 1 000 *g* for 10 min, to remove cell debris; (2) supernatant is transferred to another tube and centrifuged at 16 000 *g* for 1 h, to collect microvesicles; (3) supernatant is filtered in a 0.22 µm pore filter, to remove the remaining particles larger than 200 nm, and transferred to an ultracentrifuge tube; (4) supernatant is centrifuged at 100 000 *g* for 2 h, to pellet exosomes: (5) the pellet is resuspended in PBS to wash the exosomes; (6) finally, exosomes are centrifuged one the last time at 100 000 *g* for 2 h and at the end labelled for further incubation, characterized by Nanoparticle Tracking Analysis or resuspended in lysis buffer from the miRCURY™ RNA Isolation Kit for RNA isolation.

2.2.2. Exosomes labeling with PKH67 fluorescent probe

In order to study whether neuronal-derived exosomes were incorporated by CHME3 cells, isolated exosomes (Figure II.3) were resuspended in PBS and labeled with a PKH67 fluorescent probe, using the PKH67 Fluorescent Linker Kit (Sigma, Aldrich). Briefly, the PKH67 dye was diluted in a Diluent C, supplied with the kit. Then, equal volumes of exosomes suspension and PKH67 solution were mixed (1:1 v/v) and incubated at room temperature (RT) for 5 min. Exosomes were wash two times by adding PBS and centrifuged at 100 000 g for 70 min. In the end, exosomes were resuspended in DMEM with 1% AB/AM and CHME3 cells incubated for 24 h as described in **section 2.1.2** (Figure II.1).

2.2.3. Evaluation of exosomes concentration and particle size

To assess the concentration and size of the exosomes derived from neuroblastoma cells, isolated exosomes were resuspended in PBS and then subjected to the Nanoparticle Tracking Analysis (NTA) using the NanoSight equipment (Malvern). NTA is used to characterize nanoparticles from 30nm-1000nm dependent on sample type and it relies on direct observation and measurement of diffusion events. This particle-by-particle methodology allows high resolution results for particle size distribution and particle concentration (Filipe et al., 2010).

2.3. Evaluation of cell viability

To determine cell viability, following culture media removal, both adherent and floating cells, removed by trypsin detachment and from culture medium, respectively, were collected by centrifugation at 500 g for 5 min. Pellet was resuspended in 1% BSA in PBS and stained with phycoerythrin-conjugated annexin V (V-PE) and 7-amino-actinomycin D (7-AAD), Guava Nexin® Reagent (Milipore; MA, USA)

according to manufacturer's instruction. Stained cells were analyzed on a flow cytometer equipment (Guava easyCyte 5HT) using the Guava Nexin® Software module. Three populations of cells can be distinguished in this assay: viable cells (annexin V-PE and 7-AAD negative), early-apoptotic cells (annexin V-PE positive and 7-AAD negative) and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

2.4. Cell staining and immunocytochemistry

2.4.1. Lysosome labeling with LysoTracker™ Red DNA-99

To evaluate CHME3 cell lysosomal activity, following incubation cells were labelled at each time point with LysoTracker™ Red DND-99 fluorescent probe for 30 min. Afterwards cells were fixed with 4% (w/v) PFA in PBS and cell nuclei stained with Hoechst 33258 dye (1:1000 in PBS, Sigma-Aldrich) for 2 min. Fluorescence was visualized using a fluorescence microscope (model AxioScope.A1) coupled with and AxioCam HR camera (Carl Zeiss). UV and red fluorescence images of ten random microscopic fields were acquired per sample under 630x magnification.

2.4.2. Autophagy evaluation

For autophagy evaluation, SH-SY5Y, SH-SY5Y APP_{Swe} and CHME3 cells were fixed, permeabilized and blocked. Cells were incubated overnight at 4°C with the primary antibody rabbit anti-LC3 (1:200; Cell Signaling Technology®) prepared in antibody solution (1% BSA in PBS). Cells were washed in PBS and incubated for 2 h at RT with the secondary antibody goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen Corporation™). Cell nuclei were stained with Hoechst 33258 dye as above. UV and green fluorescence images of ten random microscopic fields were acquired per sample under 630x magnification.

2.4.3. HiPSC-derived astrocytes characterization

For the hiPSCs-derived astrocytes characterization, cells were fixed, permeabilized and blocked. Then, cells were incubated overnight at 4°C with the primary antibodies mouse anti-GFAP (1:100; Novocastro) and rabbit anti-GLT-1 (1:180; Abcam) or mouse anti-GFAP (1:100; Novocastro) and rabbit anti-S100B (1:200; Abcam) prepared in antibody solution. Cells were washed in PBS and incubated for 2 h at RT with the secondary antibodies goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 (1:1000; Invitrogen Corporation™). Cell nuclei were stained with Hoechst 33258 dye as above. UV, green and red fluorescence images of ten random microscopic fields were acquired per sample under 400x magnification.

2.5. Total RNA extraction, reverse transcription and RealTime-PCR

Determination of inflammatory genes and miRNAs expression was performed by quantitative real time-PCR (qRT-PCR). Total RNA was extracted from SH-SY5Y, SH-SY5Y APP_{Swe}, CHME3 and astrospheres using TRIzol® reagent, according to the manufacturer instructions. RNA from exosomes

was extracted using the miRCURY™ RNA Isolation Kit (Exiqon). Total RNA was quantified using Nanodrop® ND-100 Spectrophotometer (NanoDrop Technologies).

For mRNA expression, aliquots of 300 ng/µl were reverse transcribed into cDNA using GRS cDNA Synthesis Kit (GRISP, Research Solutions), under the recommended conditions. qRT-PCR was performed on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems), using a Xpert Fast SYBR (Blue) Kit (GRISP, Research Solutions). The sequences listed in Table II.2 were used as primers. qRT-PCR was performed in a 384-well plates, with each sample performed in duplicate, and under optimized conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 amplification cycles at 95°C for 5 seconds and 62°C for 30 seconds. In order to verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol (95ºC for 15 s, followed by 60ºC for 30 s and 95ºC for 15 s). As endogenous control it was used the housekeeping gene GAPDH and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Table II.2-List of primer sequences used for gene expression in qRealTime-PCR.

Gene	Sequence (5'-3')
S100B	5'-GAGAGAGGGTGACAAGCACAA-3' (fwr) 5'-GGCCATAAACTCCTGGAAGTC-3' (rev)
HMGB1	5'-CTCAGAGAGGTGGAAGACCATGT-3' (fwr) 5'-GGGATGTAGGTTTTCATTTCTCTTTC-3' (rev)
RAGE	5'-TTCACGAAGTTCCAAACAGGT-3' (fwr) 5'-GTTCTAGGACGACTGGGGTG-3' (rev)
TNF-α	5'- AACCTCCTCTCTGCCATC-3' (fwr) 5'- ATGTTCGTCCTCCTCACA-3' (rev)
Cx43	5'-ACAGCGGTTGAGTCAGCTTG-3' (fwr) 5'-GAGAGATGGGGAAGGACTTGT-3' (rev)
IL-10	5'- CCTGGAGGAGGTGATGCCCCA-3' (fwr) 5'- CCTGCTCCACGGCCTTGCTC-3' (rev)
GAPDH	5'- CGCTCTCTGCTCCTCCTGTT-3' (fwr) 5'- CCATGGTGTCTGAGCGATGT-3' (rev)

Expression of inflammatory miRNAs was also performed by qRT-PCR After RNA quantification, cDNA synthesis was performed using with the Universal cDNA Synthesis Kit II (Exiqon), using 10 ng/µl of total RNA and 0,5 µl of mRNAUniSp6 according to the following protocol: 60 min at 42°C followed by heat-inactivation of the reverse transcriptase for 5 min at 95°C. For miRNA quantification, the SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents Kit (Applied Biosystems) was used in combination with pre-designed primers (Exiqon) listed in Table II.3. The reaction conditions consisted of polymerase activation/denaturation at 95°C for 10 min, followed by 50 amplification cycles at 95°C for 10 seconds and 60°C for 1 min (ramp-rate of 1.6°/second). Quantification of target miRNAs was made in comparison to the reference gene (U6) and spike-in UniSp6, and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Table II.3-List of primer sequences used for microRNA expression in qRealTime-PCR.

miRNAs	Sequence (5'-3')
has-miR-124-3p	5'-UAAGGCACGCGGUGAAUGCC-3'
has-miR-155-5p	5'- UUAAUGCUAAUCGUGAUAGGGGU-3'
has-miR-125b-5p	5'- UCCCUGAGACCCUAACUUGUGA-3'
has-miR-21-5p	5'- UAGCUUAUCAGACUGAUGUUGA-3'
has-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'
U6	Reference gene
UniSp6	Spike in quality control gene

2.6. Statistical analysis

Results were expressed as mean ± SEM. Differences between groups were determined by one-way or two-way ANOVA and differences between parameters were determined by one-tailed Student's t-test, as appropriated, followed by multiple comparisons Bonferroni *post hoc* correction. *p* values less than 0.05 were considered statistically significant. Statistical analysis was made using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA).

III. Results

1. Characterization of SH-SY5Y and SH-SY5Y APP_{Swe} cells and their derived exosomes

SH-SY5Y cell line is an immortalized and proliferative cell line obtained from a bone marrow biopsy of a neuroblastoma patient. It is frequently used in *in vitro* studies of neurodegenerative diseases due to their ability and potential to differentiate into a neuronal phenotype (Forster et al., 2016). The characterization of these cells was recently performed in our group showing that SH-SY5Y APP_{Swe} cells express, intracellularly, immature APP695 with an increased signal over that of SH-SY5Y cells, and secrete elevated sAPPα levels. Additionally, concentration of secreted Aβ species, measured by ELISA, was higher in SH-SY5Y APP_{Swe} cells, namely for the $Aβ_{1-40,}$ when compared to SH-SY5Y cells. Here, we decided to further characterize these cells by assessing SH-SY5Y and SH-SY5Y APP_{Swe} inflammatory-related markers, such as miRNAs and cytokine mRNA expression, both in cells and in their derived exosomes in order to clarify their distinct phenotype, before exosome incubation with human CHME3 microglial cells.

1.1. APP Swedish mutation does not affect SH-SY5Y viability

Having identified the different expression of APP and $Aβ_{1-40}$ by SH-SY5Y APP_{Swe} cells we next assessed whether such mutation could affect cell viability. Using the Guava Nexin® Reagent, as described in Methods section, we observed that SH-SY5Y APP_{Swe} cells showed a slight and not significant reduction in cell viability. Cells also revealed a small and not significant increase in early apoptosis measured by externalization of phosphatidylserine (Figure III.1). These results indicate that APP_{Swe} mutation does not cause toxicity in neuroblastoma cell line, as expected.

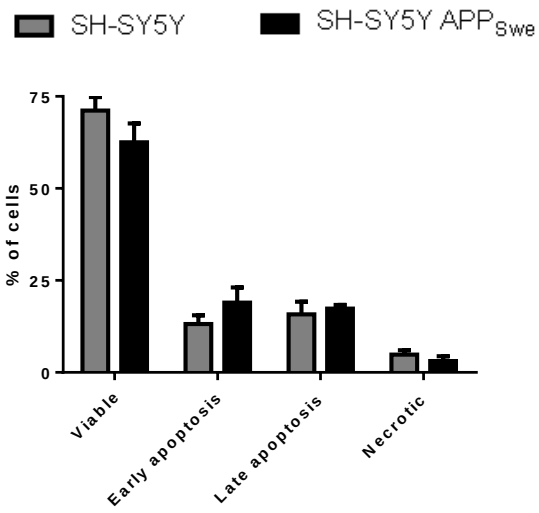

Figure III.1-Evaluation of cell viability/cell death in SH-SY5Y and SH-SY5Y APPSwe cells. Human neuroblastoma SH-SY5Y and SH-SY5Y APPSwe cells were differentiated using retinoic acid (10 µM) for 7 days, and then cell viability/cell death was evaluated at day 9 using the Guava Nexin® Reagent. Four populations of cells were distinguished: viable cells (annexin V-PE and 7-AAD negative), early-apoptotic cells (annexin V-PE positive and 7-AAD negative), cells in late stages of apoptosis or dead (annexin V-PE and 7-AAD positive) and necrotic/debries (annexin V-PE negative and 7-AAD positive).Graph bars represent mean ± SEM from six independent experiments.

1.2. Exosomes from SH-SY5Y and SH-SY5Y APP_{Swe} cells show similar diameter size

Next, we isolated exosomes from the extracellular media of SH-SY5Y and SH-SY5Y APP_{Swe} cells and analyzed their number and diameter size using the NTA technique, as mentioned in Methods. As depicted in Figure III.2A, exosomes derived from SH-SY5Y APP_{Swe} cells show a slight, but not significant, decrease of particle concentration when compared to SH-SY5Y-derived exosomes. Although no changes were observed in the size of exosome diameter average between SH-SY5Y and SH-SY5Y APP_{Swe} cells (Figure III.2B), the histogram analysis shows that exosomes from SH-SY5Y have a particle size ranging from 60 to 380 nm, while exosomes from SH-SY5Y APP_{Swe} are distributed from 20 to 320 nm (Figure III.2C). In more detail, exosomes from SH-SY5Y have a higher distribution between 140-340 nm with 3 peaks (~180, 240 and 290 nm), while exosomes from SH-SY5Y APP_{Swe} cells show a prevalent distribution between 130-210 nm, with a maximal peak near ~180 nm.

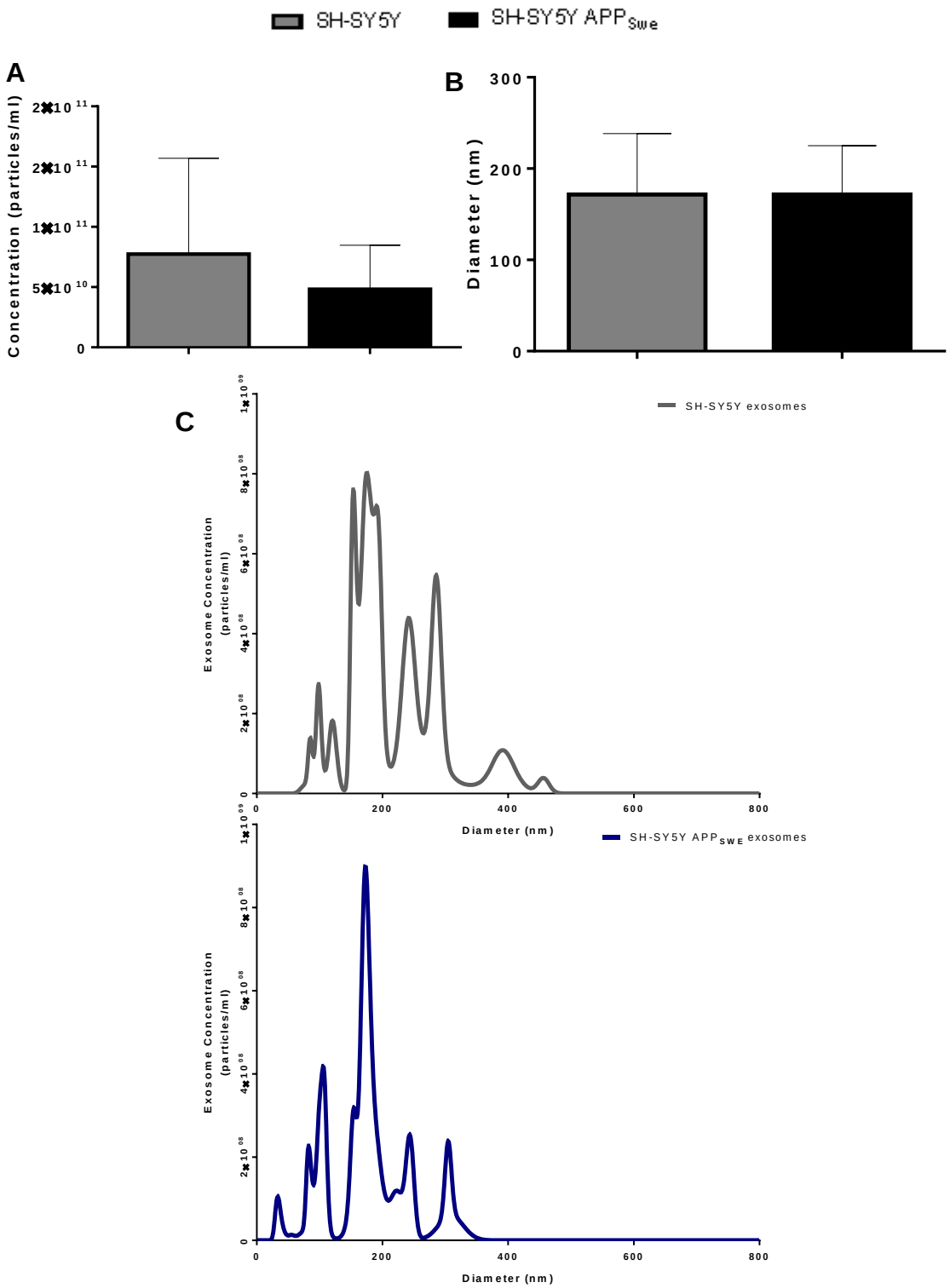

Figure III.2-Size and particle concentration of exosomes derived from neuroblastoma cells. Human neuroblastoma SH-SY5Y and SH-SY5Y APPSwe cells were differentiated using retinoic acid (10 µM) for 7 days and then exosomes isolated at day 9 by ultracentrifugation. Exosomes characterization was performed by Nanoparticle Tracking Analysis (NTA) using the NanoSight. (A) Results of particle concentration are expressed as particles/ml. (B) Graph bars represent average particle size expressed in nanometers (nm). (C) Histogram represent exosomes concentration (particles/ml) along diameter (nm) variation. Results of graph A and B are represented as mean (± SEM) from at least four independent experiments.

1.3. SH-SY5Y APP_{Swe} cells show upregulated inflammatory-associated miRNAs, from which some of them are transferred into their derived exosomes

Inflamma-miRs have gained special attention due to their ability of modulating cell activation and consequently neuroinflammation, as previously mentioned (Ponomarev et al., 2013; Femminella et al., 2015). Therefore, we assessed the expression of several miRNAs involved in inflammation in both control and AD cells, as well as in their exosomes. As shown in Figure III.3 SH-SY5Y APP_{Swe} cells express significantly higher levels of miR-124 (3.6-fold, $p<0.05$), miR-155 (9.0-fold, $p<0.05$), miR-146a (6.5-fold, $p<0.01$), miR-21 (7.9-fold, $p<0.05$), and miR-125b (6.9-fold, $p<0.01$). Interestingly, exosomes derived from SH-SY5Y APP_{Swe} cells recapitulated the original cell of in terms of increased miR-124 (11.6-fold, $p<0.05$), miR-21 (3.4-fold, $p<0.05$) and miR-125b (3.6-fold, $p<0.05$).

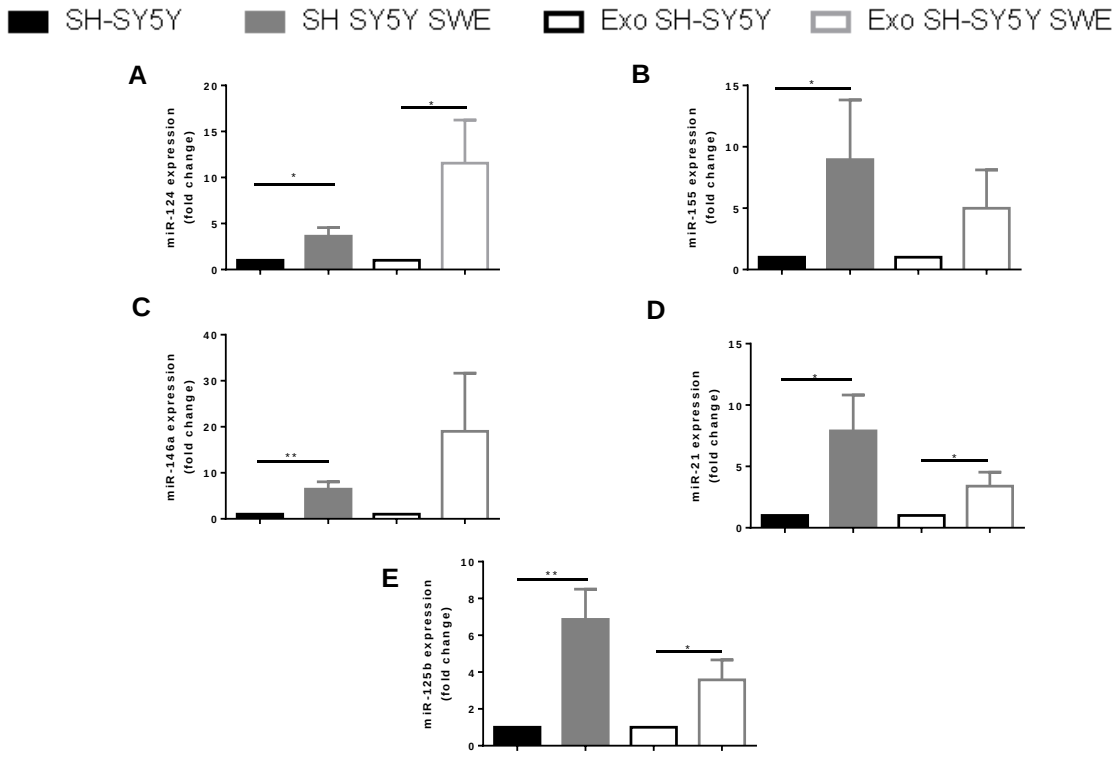

Figure III.3-Expression of microRNA (miR)-124, miR-155, miR-146a, miR-21 and miR-125b in SH-SY5Y and SH-SY5Y APPSwe and their derived exosomes. Human neuroblastoma SH-SY5Y and SH-SY5Y APPSwe cells were differentiated using retinoic acid (10 µM) for 7 days and then exosomes isolated at day 9 by ultracentrifugation. MicroRNA expression in cells and exosomes was evaluated by quantitative Real-Time PCR (qRT-PCR), as indicated in methods section. (A-E) Relative miR-124, miR-155, miR-146a, miR-21, miR-125b levels were determined by qRT-PCR in total RNA. Results are represented by mean (± SEM) from 6 independent experiments. Fold change was calculated relatively to SH-SY5Y cells or SH-SY5Y-derived exosomes. *$p<0.05$ and **$p<0.01$ *vs* SH-SY5Y or *vs* SH-SY5Y exosomes.

1.4. SH-SY5Y APP_{Swe} cells express elevated DAMPs and cytokines that are not significantly reflected in their exosomes

We first assessed if SH-SY5Y APP_{Swe} cells and their exosomes differently express common inflammatory mediators, such as S100B and HMGB1 when compared to SH-SY5Y cells. We found that

mRNA expression levels of S100B and HMGB1 in SH-SY5Y APP_{Swe} cells were higher than those in SH-SY5Y cells (6.3-fold and 1.9-fold, respectively, $p<0.05$), as depicted in Figure III.4A and 4B. Next we evaluated RAGE mRNA expression, which is a S100B and HMGB1 receptor, as well as an Aβ peptide transporter (Yan et al., 2009). As observed for S100B and HMGB1 gene expression, also RAGE was upregulated in SH-SY5Y APP_{Swe} cells relatively to SH-SY5Y cells (2.2-fold, $p<0.05$) (Figure III.4C). Interestingly, when the expression of such molecules was evaluated in cell-derived exosomes, we found a similar not significant tendency for S100B and RAGE, but we could not detect the presence of HMGB1 mRNA, as shown in Figure III.4.B. These results indicate that HMGB1 mRNA is not a component of exosomal cargo.

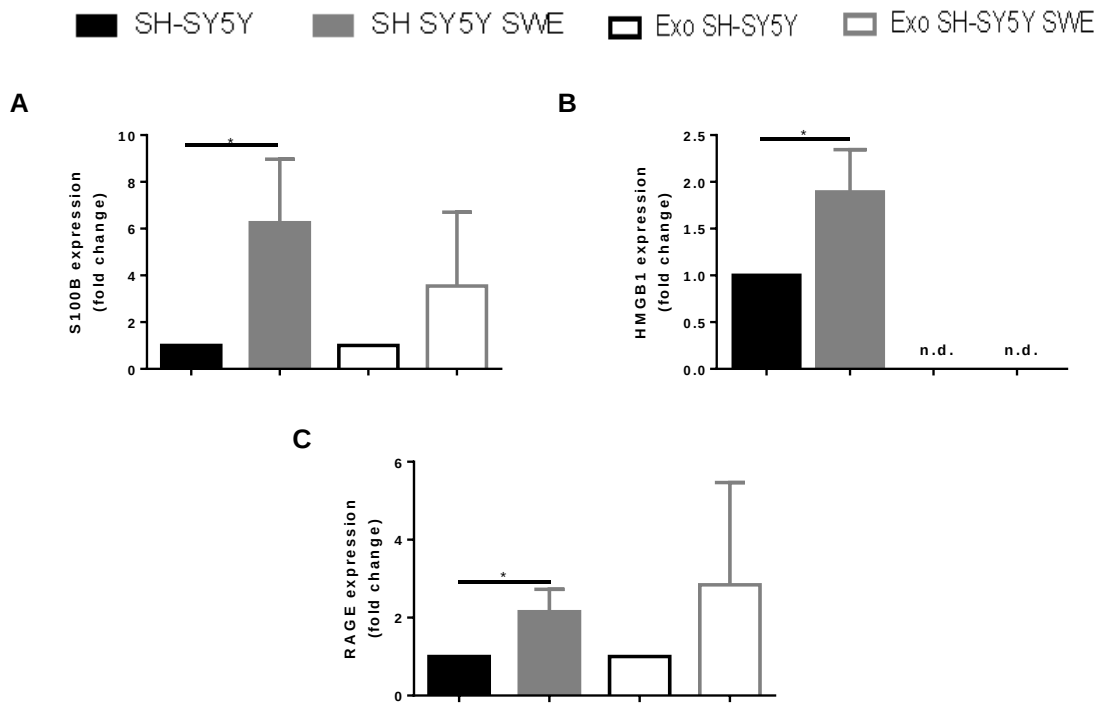

Figure III.4-SH-SY5Y APPSwe cells express increased levels of S100B and HMGB1, as well as of their receptor RAGE. Human neuroblastoma SH-SY5Y and SH-SY5Y APPSwe cells were differentiated using retinoic acid (10 µM) for 7 days and then exosomes isolated at day 9 by ultracentrifugation. mRNA expression of both SH-SY5Y and SH-SY5Y APP_{Swe} cells and respective exosomes was determined by quantitative Real-Time PCR in total RNA. (A) Relative mRNA expression of S100 calcium-binding protein B (S100B), (B) High-mobility group box 1 (HMGB1) and (C) Receptor for advanced glycation end-products (RAGE) in cells and their derived exosomes. Results are represented by mean (± SEM) from 6 independent experiments. Fold change was calculated relatively to the SH-SY5Y cells or SH-SY5Y-derived exosomes. *$p<0.05$ *vs* SH-SY5Y. Abbreviaton: not detected (n.d).

Then, we decided to assess the neuronal mRNA expression of two specific inflammatory cytokines, i.e. TNF-α, a first line pro-inflammatory cytokine, and IL-10, a potent anti-inflammatory cytokine, either in cells or in the released exosomes. As indicated in Figure III.5A and 5B, SH-SY5Y APP_{Swe} cells showed a significant upregulation of both TNF-α (4.6-fold, $p<0.05$) and IL-10 (2.2-fold, $p<0.05$), when compared to SH-SY5Y ones. However, exosomes released from SH-SY5Y APP_{Swe} cells

expressed lower mRNA levels of TNF-α than those from SH-SY5Y cells (0.01-fold, $p<0.01$), while similar levels were observed for IL-10. These results indicate that increased cellular TNF-α and IL-10 is not recapitulated in SH-SY5Y APP_{Swe}-derived exosomes.

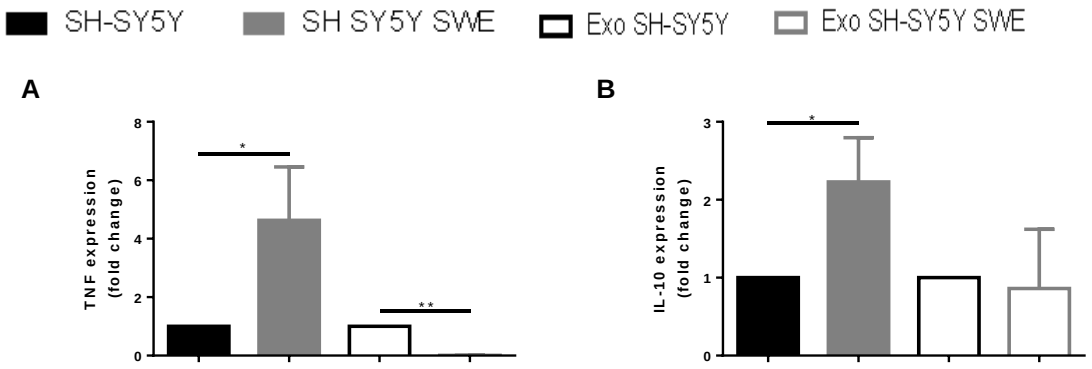

Figure III.5-TNF-α and IL-10 mRNA expression is increased in SH-SY5Y APPSwe cells. Human neuroblastoma SH-SY5Y and SH-SY5Y APPSwe cells were differentiated using retinoic acid (10 µM) for 7 days and then exosomes isolated at day 9 by ultracentrifugation. mRNA expression of both SH-SY5Y and SH-SY5Y APP_{Swe} cells and respective exosomes was determined by quantitative Real-Time PCR in total RNA. (A) Relative mRNA expression of Tumor necrosis factor-α (TNF-α) and (B) Interleukin-10 (IL-10) in cells and its derived exosomes. Results are represented by mean (± SEM) from 6 independent experiments. Fold change was calculated relatively to SH-SY5Y cells or SH-SY5Y exosomes. *$p<0.05$ and **$p<0.01$ *vs* SH-SY5Y or *vs* SH-SY5Y-derived exosomes.

2. Evaluation of human microglia CHME3 reactivity to exosomes released by SH-SY5Y and SH-SY5Y APP_{Swe} cells

Having characterized the SH-SY5Y APPSwe cells and their exosomes relatively to specific changes in miRNA and cytokine mRNAs expression, we next decided to evaluate whether such AD modified exosomes were able to induce alterations in microglia activation and dysfunction, by using the human CHME3 cell line incubated with exosomes delivered by SH-SY5Y or SH-SY5Y APP_{Swe} cells. To address this question the CHME3 cell line was incubated with SH-SY5Y and SH-SY5Y APP_{Swe} derived exosomes and evaluated at the end of the 24 h incubation period. Then, exosomes were isolated and microglia were left for an additional 24 h to assess their ability to recover after interaction with exosomes.

2.1. Exosomes from SH-SY5Y and SH-SY5Y APP_{Swe} cells are collected by CHME3 microglia and co-localize with lysosomes

To determine how CHME3 microglial function was modified after interaction with exosomes derived from both neuroblastoma cell lines, we started by assessing if microglia was able to uptake such extracellular vesicles. Indeed, previous data from the group using an *in vitro* ALS model showed that mouse microglial cell line N9 was able to uptake exosomes delivered by SOD1 mutated motor neurons (Pinto et al., 2017).

As shown in Figure III.6, exosomes from SH-SY5Y APP_{Swe} and SH-SY5Y were similarly internalized by CHME3 upon 24 h incubation, based on the density of PKH67 (green) labeled exosomes inside the cells, namely at the perinuclear area, where staining for lysosomes were particular evident. However, we noticed the disappearance of the staining at 48 h by the additional 24 h period of incubation after isolation of exosomes and cell medium replacement, together with the loss of lysosomal staining.

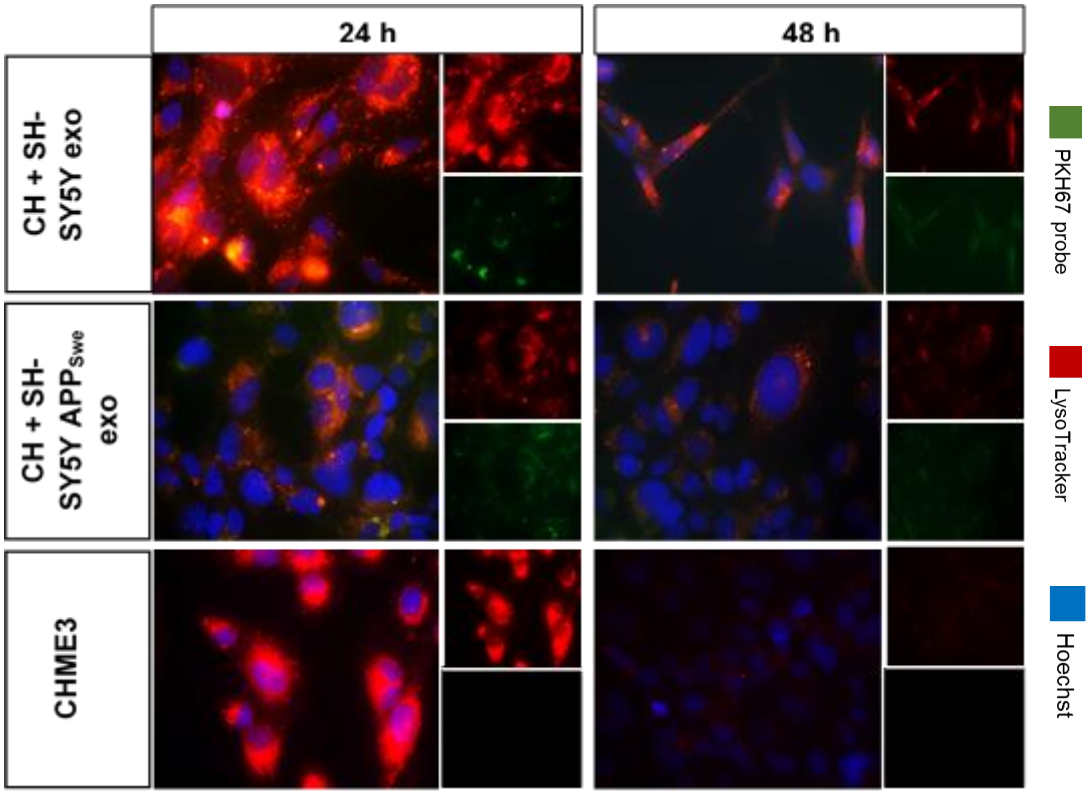

Figure III.6- SH-SY5Y and SH-SY5Y APP_{Swe}-derived exosomes were similarly internalized by CHME3 microglia after 24 h incubation, and degraded at 48 h by an additional 24 h period of microglia incubation in a new medium. CHME3 microglial cells were incubated with SH-SY5Y and SH-SY5Y APP_{Swe}-derived exosomes, as described in Methods section. Exosomal distribution in microglia was identified by labeling of exosomes with the fluorescent PKH67 probe previous to incubation. CHME3 lysosomal activity was identified by LysoTracker™. Cell nuclei were stained with Hoechst 33258 dye. Representative images of one experiment are shown for each incubation time period (24 and 48 h).

When we quantified the PKH67 fluorescence intensity (Figure III.7) we observed a slight staining decrease, although not significant, in CHME3 cells incubated with SH-SY5Y APP_{Swe}-derived exosomes at 24 h, but even more obvious at the end of the 48 h incubation. Later, we confirmed such finding by measuring the exosomal area. Actually, in these conditions we were able to establish that a reduction had occurred (0.80-fold, $p<0.05$).

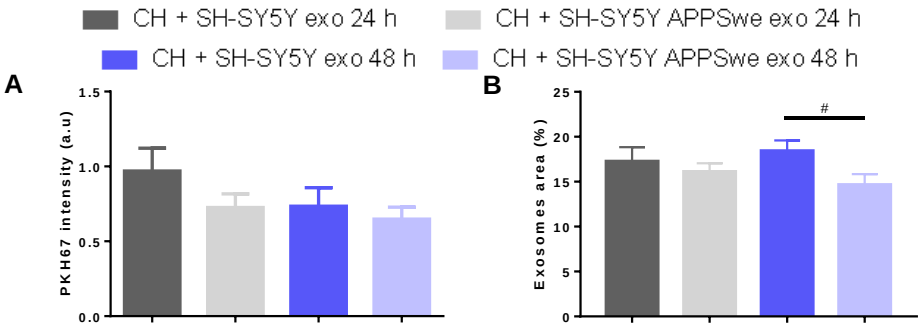

Figure III.7-SH-SY5Y APP$_{Swe}$-derived exosomes area was reduced at 48h in CHME3 cells. (A) Fluorescence intensity of PKH67 labeled exosomes after internalization by CHME3 microglia. (B) Exosomal area determined by ImageJ software. Results are represented as mean (± SEM) of at least four independent experiments. $^{\#}p<0.01$ *vs.* CHME3 treated with SH-SY5Y-derived exosomes.

Since it has been described that exosome membrane proteins/cargo may be trapped in lysosomes following cellular endocytosis (Tian et al., 2010), we next evaluated whether this could be occurring in our culture system. So, we assessed the colocalization of the labeled exosomes in CHME3 cells and the lysosomes stained with LysoTracker™. As observed in Figure III.6/III.8, exosomes detected by PKH67 staining almost completely co-localized with lysosomes (red) at 24 h incubation. However, a reduction in the co-localization was noticed for the exosomes derived from SH-SY5Y APP$_{Swe}$ cells relatively to those of SH-SY5Y after the 48 h period (0.85-fold, $p<0.05$), suggesting a faster degradation of such AD exosomes by the lysosomes. Curiously, lysosomal staining upon 48 h incubation, was also significantly decreased in CHME3 exposed to SH-SY5Y APP$_{Swe}$-derived exosomes (0.77-fold, $p<0.01$), which may indicate a compromised lysosomal activity and degradation.

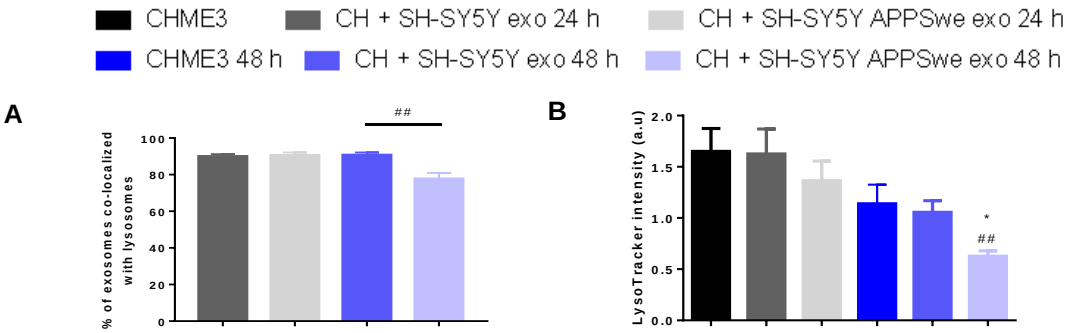

Figure III.8- Co-localization of SH-SY5Y APP$_{Swe}$-derived exosomes with lysosomes in CHME3 cells decrease at 48 h. (A) Co-localization results were obtained by ImageJ software. (B) CHME3 lysosomes were identified by labeling cells with LysoTracker™ dye. LysoTracker intensity was quantified using the ImageJ software. Results are represented as mean (± SEM) of at least four independent experiments. $^{*}p<0.05$ *vs.* CHME3 control cells for each time period, $^{\#\#}p<0.01$ *vs.* CHME3 treated with SH-SY5Y-derived exosomes.

2.2. Treatment of CHME3 microglia with SH-SY5Y APP$_{Swe}$-derived exosomes does not differently compromise autophagy but reduces cell viability in a small extent

Having verified a decreased lysosomal activity, we next assessed autophagy, a process of lysosome-dependent intracellular degradation, described to be dysfunctional in AD (Orr and Oddo,

2013). We used the evaluation of LC3 immunostaining. LC3 is a soluble protein that is distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, the cytosolic form of LC3 (LC3-II) is recruited to the autophagosomal membrane. Later, autophagosomes fuse with lysosomes and LC3-II is degraded, which reflects the autophagic activity (Tanida et al., 2008). As shown in Figure III.9A, at 24 h of microglia incubation we have not observed differences between CHME3 cells not incubated or incubated with neuroblastoma-derived exosomes. In contrast, at the end of the 24 h incubation plus 24 h recovery, while CHME3 cells showed an increased trend in the LC3 staining, exosomal internalization from both SH-SY5Y- and SH-SY5Y APP_{Swe} cells determined a significant decrease of the LC3 fluorescence intensity. Indeed, when we evaluated the intensity of LC3 in the cells (Figure III.9B) we observed no changes after 24 h incubation, but a marked decrease at 48h for cells exposed to exosomes (p<0.01). However, no differences were observed between cells incubated with SH-SY5Y- or SH-SY5Y APP_{Swe}-derived exosomes.

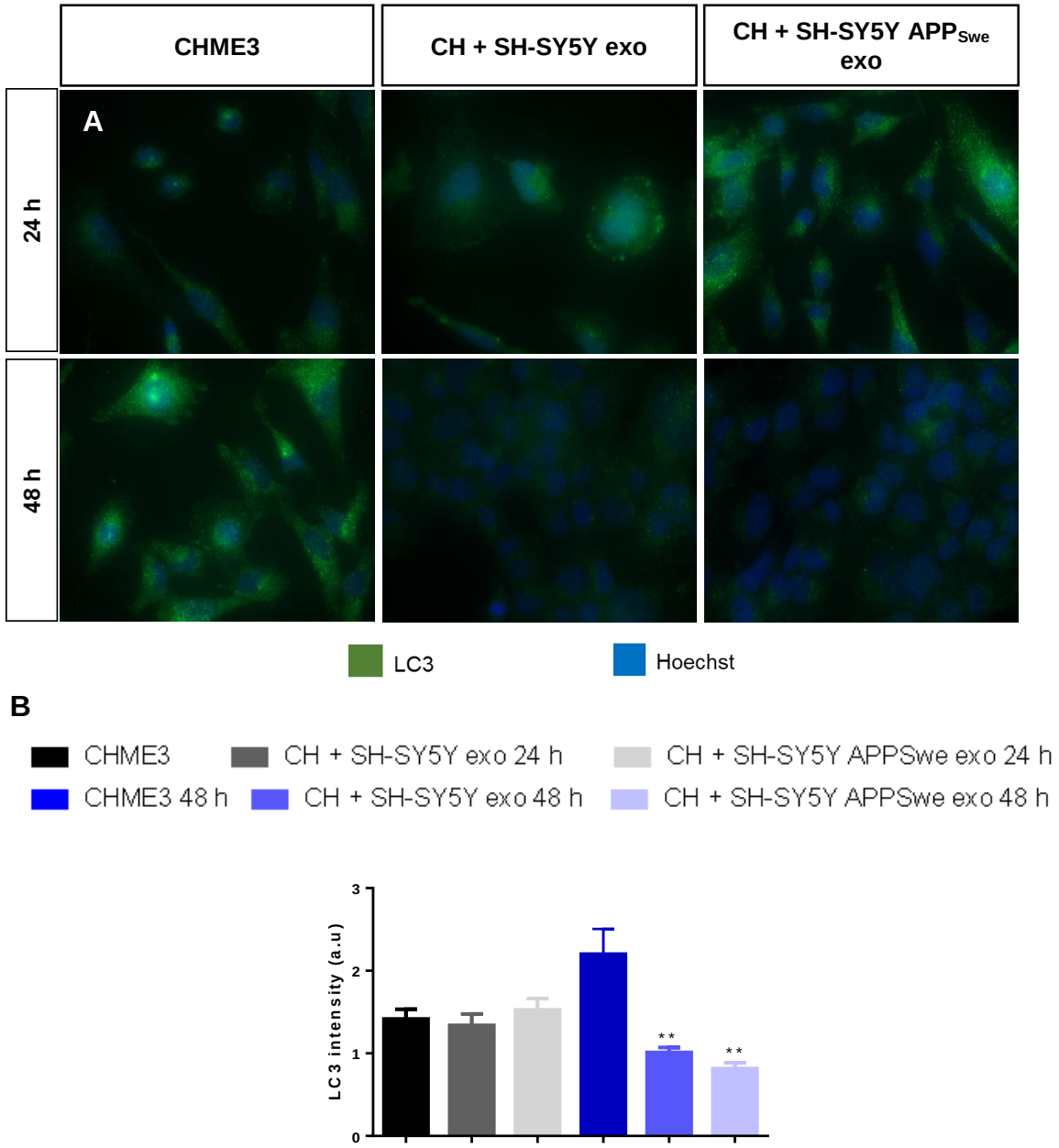

Figure III.9- Fluorescence intensity of the autophagic marker LC3 in CHME3 microglial cells is reduced after 24 h exposure to both SH-SY5Y- and SH-SY5Y APP$_{Swe}$-derived exosomes followed by a period of 24 h recovery. CHME3 microglial cells were incubated with SH-SY5Y and SH-SY5Y APP$_{Swe}$-derived exosomes, as described in Methods section. (A) Autophagy was evaluated by immunocytochemistry using rabbit anti-Microtubule-associated protein 1A/1B-light chain 3 (LC3) antibody, followed by a fluorescent-labeled secondary antibody. Representative images of one experiment are shown for each time point (24 and 48 h). (B) The fluorescent intensity of cellular LC3 was quantified using the ImageJ software. Results are represented as mean (± SEM) of at least four independent experiments. ***p*<0.01 *vs.* control CHME3 cells at 24 h and 48 h time points.

Finally, we assessed if exosomes were able to compromise CHME3 cell viability, using the Guava Nexin® Reagent. In Figure III.10 it is shown that CHME3 microglia exposed for 24 h to exosomes from SH-SY5Y APP$_{Swe}$ cells only slightly decrease their viability when compared to non-treated CHME3

cells (0.95-fold, **p*<0.05). Curiously, when cell death was evaluated at the end of the 48 h incubation, cells that were treated with SH-SY5Y APP_{Swe}–derived exosomes showed a decreased cell viability, when compared to cells exposed to SH-SY5Y-derived exosomes (0.93-fold, *p*<0.05), possibly as a result of the increase, although not significant, in cells suffering late apoptosis and necrosis. These results indicate that, in our incubation conditions, exposure of CHME3 to SH-SY5Y APP_{Swe}–derived exosomes do not have a real toxic effect and support that results indicated below are not due to cell demise.

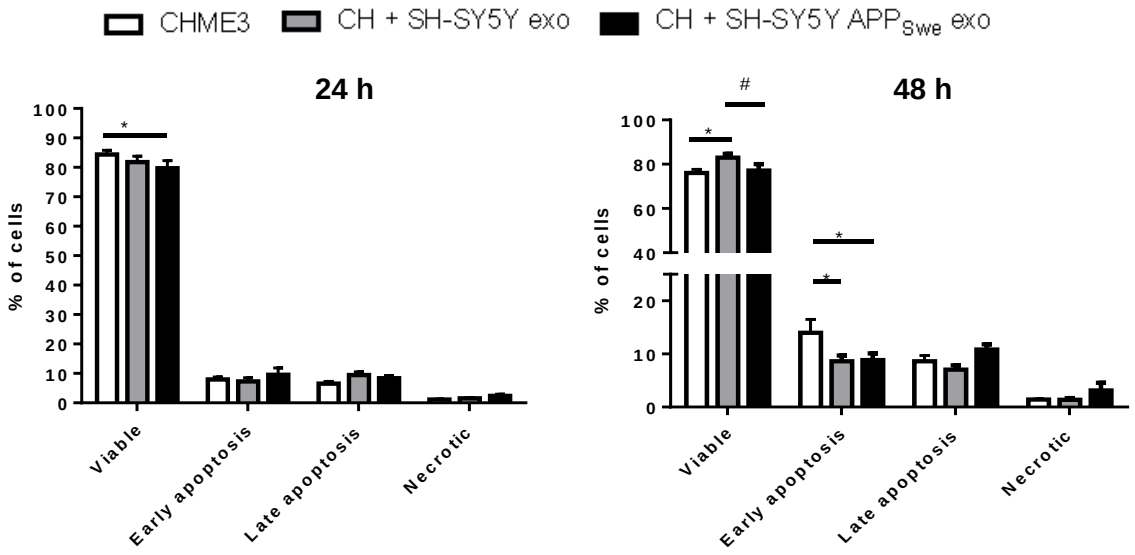

Figure III.10-Effect of exosomes delivered by SH-SY5Y and SH-SY5Y APPSwe cells on the viability of CHME3 microglial cells. CHME3 cell viability was evalutated at each time point (24 h and 48 h) using Guava Nexin® Reagent. Four populations of cells were distinguished: viable cells (annexin V-PE and 7-AAD negative), early-apoptotic cells (annexin V-PE positive and 7-AAD negative), cells in late stages of apoptosis or dead (annexin V-PE and 7-AAD positive) and necrotic/debries (annexin V-PE negative and 7-AAD positive).Results are percentage of cells ± SEM from six independent experiments for each group. **p*<0.05 *vs.* CHME3 cells for each time point, $^{\#}p$<0.05 *vs.* CHME3 treated with SH-SY5Y-derived exosomes.

2.3. SH-SY5Y APP_{Swe}-derived exosomes do not induce significant changes of inflammatory-associated miRNAs in CHME3 microglial cells, but determine an increased expression of miR-21 in their derived exosomes, as compared with the exosomes from SH-SY5Y cells

Neuroinflammation is a known hallmark in AD pathology (Harry and Kraft, 2008; Morales et al., 2014). MiRNAs play an active role in the molecular pathogenesis of AD namely acting as mediators of the inflammatory response (Goodall et al., 2013). Therefore, we next evaluated whether microglia response in terms of miRNA expression and their release via exosomes differ when cells are treated with the SH-SY5Y or SH-SY5Y APP_{Swe}–derived exosomes.

First, it deserves to be noted that we were not able to find significant alterations in CHME3 miRNA profile after treatment with either SH-SY5Y or SH-SY5Y APP_{Swe} cells. In some cases, the variations were elevated and neutralized the resultant effects. However, it seems interesting to notice that at the end of 48 h incubation, 24 h with exosomes and 24 h of recovery, microglia initially treated with SH-SY5Y APP_{Swe}-derived exosomes seem to have higher propensity to evidence elevated miR-21 levels at both periods (3.0-fold at 24 h; 3.7-fold at 48 h) (Figure III.11). As, aforementioned, this miR-21 was found increased in SH-SY5Y APP_{Swe} and in their derived exosomes (Figure III.3). Accordingly, exosomes

isolated from CHME3 after treatment with SH-SY5Y APP_{Swe}-derived exosomes for 24 h and left to recovery for an additional 24 h also showed increased miR-21 levels if compared with the microglia treated with SH-SY5Y-derived exosomes, but not with non-treated cells. This finding is important since extracellular vesicles containing miR-21 were shown to be neurotoxic (Yelamanchili et al., 2015). Finally, miR-125b was also observed tentatively decreased in CHME3 cells treated with SH-SY5Y APP_{Swe}-derived exosomes at 24 h and after the recovery period.

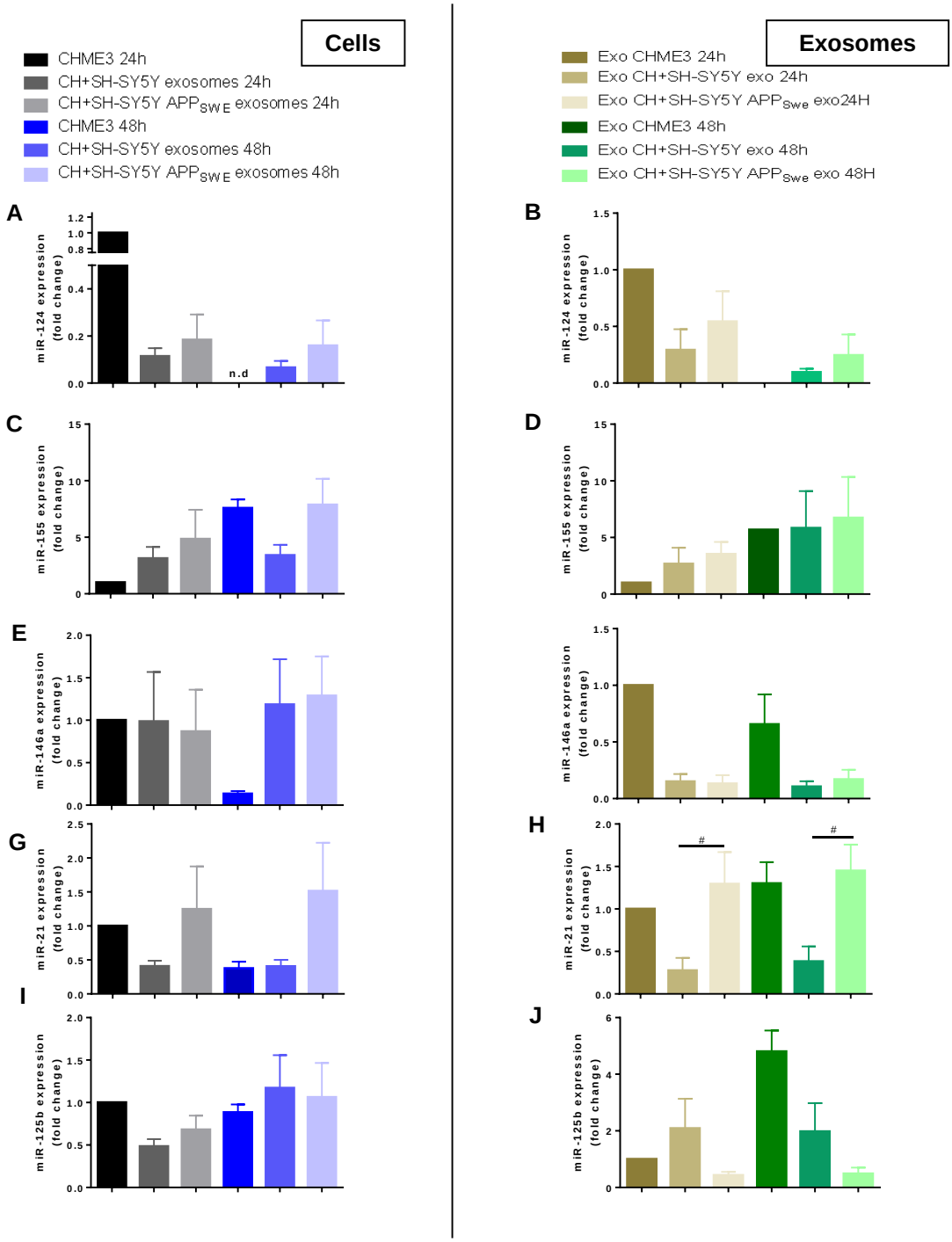

Figure III.11- Exosomes from CHME3 microglia treated with SH-SY5Y APPSwe display increased levels of miR-21 when compared with cells incubated with SH-SY5Y-derived exosomes, although no changes were noticeable in the cells. MicroRNA expression in CHME3 cells was evaluated by quantitative Real-Time PCR (qRT-PCR), as indicated in methods section. CHME3 microglia cells were incubated for 24 h with exosomes from both SH-SY5Y and SH-SY5Y APP_{Swe} cells and left to recovery for additional 24 h. Exosomes were isolated by differential centrifugation. (A-I and B-J). Relative miR-124, miR-155, miR-146a, miR-21 and miR-125b levels were determined in cells and in their exosomes by qRT-PCR in total RNA. Results are represented as mean (± SEM) from 6 independent experiments and fold change are relatively to 24 h non-treated CHME3 cells. $^{\#}p<0.01$ *vs*. CHME3 exposed to SH-SY5Y-derived exosomes. Abbreviation: not detected (n.d).

2.4. SH-SY5Y APP_{Swe}-derived exosomes trigger CHME3 increased expression of alarmins and cytokines that is not recapitulated in derived exosomes

In addition to inflammatory-related microRNAs, microglia under pathological conditions exhibit increased levels of pro-inflammatory cytokines and chemokines. Activation of receptors on microglia surface triggers a variety of intracellular signaling with the production of pro-inflammatory mediators, such as TNF-α (Cunha et al., 2016). On the other hand, DAMPs (e.g. HMGB1 and S100B) are released into the extracellular space triggering the engagement of the receptor RAGE (Buhimschi et al., 2009). Thus, to confirm the activation of microglia exposed to SH-SY5Y and SH-SY5Y APP_{Swe}-derived exosomes, we investigated the mRNA expression of these inflammatory-related molecules in CHME3 microglia and in their respective exosomes. As shown in Figure III.12, DAMPs were increased in CHME3 incubated with SH-SY5Y APP_{Swe}–derived exosomes, either for 24 h or after the 24 h additional period (S100B, 2.0- and 2.4-fold respectively, $p<0.05$; HMGB1, 16.6– and 13.9-fold respectively, $p<0.01$ and 0.05, respectively) when compared with those treated with exosomes from SH-SY5Y cells. However, CHME3-derived exosomes showed no differences in S100B mRNA expression when results from exposure to SH-SY5Y and to SH-SY5Y APP_{Swe}–derived exosomes were compared (Figure III.12B). As previously commented, we could not detect any HMGB1 mRNA expression in exosomes from CHME3 cells (Figure III.12D). Having observed increased cellular levels of S100B and HMGB1 we antecipated that the expression of the receptor RAGE would be elevated, as well. Indeed, in Figure III.12E, it can be observed that RAGE mRNA expression is increased in CHME3 cells exposed to SH-SY5Y APP_{Swe}–derived exosomes for 24 h as compared with non-treated cells (2.7-fold, $p<0.05$), an effect that was lost after the 24 h recovery (3.6-fold, not significant). No changes were noticed in CHME3-derived exosomes (Figure III.12F). Next we assessed the mRNA expression of inflammation-related cytokines. In Figure III.12G, TNF-α expression was significantly upregulated in CHME3 cells treated with SH-SY5Y APP_{Swe}-derived exosomes at 24 h and after the period recovery at 48 h (15.3- and 6.1-fold, respectively, $p<0.05$), when compared to CHME3 cells exposed to exosomes from SH-SY5Y. Again, no significant differences were noticed for exosomes released from microglial cells. Similarly to TNF-α, IL-10 expression was also significantly upregulated in microglia, but only at 24 h incubation with SH-SY5Y APP_{Swe}–derived exosomes when compared to CHME3 exposed to exosomes from SH-SY5Y cells (5.3-fold, $p<0.05$) (Figure III.12I). When exosomes released from microglia were evaluated, we observed that the expression of IL-10 was decreased when derived from CHME3 cells treated for 24 h with SH-SY5Y APP_{Swe}–derived exosomes (1-7-fold $p<0.01$) as compared with exosomes resultant from CHME3 cells treated for 24 h with SH-SY5Y–derived exosomes (2.6-fold). In any case, incubation of exosomes from the neuroblastoma cell lines with microglia determined an increased expression of IL-10 in their derived exosomes ($p<0.01$).

Overall, CHME3 cells exposed to SH-SY5Y APP_{Swe}-derived exosomes express increased levels of alarmin and cytokine mRNAs, from which only IL-10 is recapitulated in their derived exosomes, suggesting that these mRNAs are not significantly transported in exosomes, at least in our experimental models.

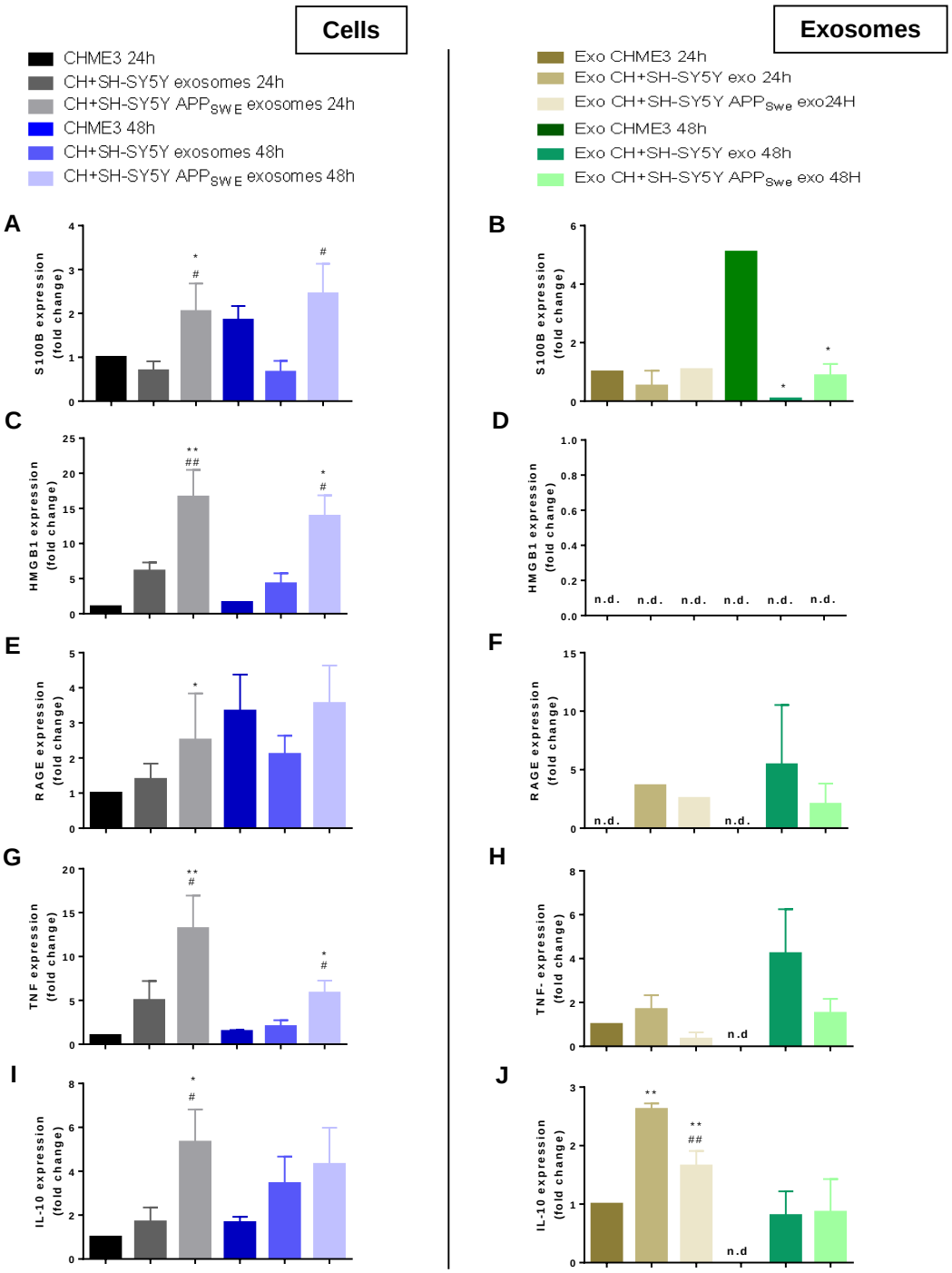

Figure III.12- CHME3 microglia treated for 24 h with SH-SY5Y APPSwe-derived exosomes show upregulation of inflammatory-related molecules, from which only IL-10 is recapitulated in the secreted exosomes. mRNA expression in CHME3 cells was evaluated by quantitative Real-Time PCR (qRT-PCR), as indicated in methods section. CHME3 microglia cells were incubated for 24 and 48h with exosomes from both SH-SY5Y and SH-SY5Y APP_{Swe} cells. Exosomes were isolated by differential centrifugation. (A-I and B-J) Relative S100 calcium-binding protein B (S100B), High-mobility group box 1 (HMGB1) and Receptor for advanced glycation end-products (RAGE), Tumor necrosis factor-α (TNF-α) and Interleukin-10 (IL-10) levels were determined in cells and in their exosomes by qRT-PCR in total RNA. Results are represented as mean (± SEM) from 6 independent experiments and fold change are relatively to 24h non-treated CHME3 cells.*p<0.05 and **p<0.01 *vs* control CHME3 cells for each time point, $^{\#}p$<0.05 and $^{\#\#}p$<0.01 *vs* CHME3 exposed to SH-SY5Y-derived exosomes. Abbreviation: not detected (n.d).

3. Dissecting the profile of astrocytes derived from iPSCs of AD patients

iPSCs have gain special attention in scientific community due to their potential to generate neurons and glial cells from healthy individuals or patients, namely those with neurodegenerative diseases, including AD (Yang et al., 2016). Moreover, iPSCs have the tremendous potential to clarify specific human pathological mechanisms and to allow the study of both sporadic and familiar forms, which may fill the gap between *in vitro*/in *vivo* models and the human response (Mungenast et al., 2016). Thus, we characterized the phenotype of astrocytes derived from AD-iPSCs bearing the PSEN1ΔE9 mutation in order to identify differences from astrocytes differentiated from healthy-iPSCs. We also assessed the same parameters in isogenic cells in which the mutation was corrected to confirm (or not) that the mutation was the only risk for the development of AD pathogenesis. Since each cell line was derived from a different individual with possible inter-individual variances, we have decided to represent the preliminary data separately for each cell line and not perform the average for each condition (i.e. control, AD, AD isogenic).

3.1. Astrocytes derived from AD-iPSCs do not show loss of cell viability

iPSCs-derived astrocytes were cultured for 1 week and cell viability of control, AD and respectively isogenic cells were measured using the Guava Nexin® Reagent. As shown in Figure III.13, although with a great variability between experiments, we could not see any differences in cell viability for each type of cell death, when control, AD and isogenic AD astrocytes were compared, suggesting that AD PSEN1ΔE9 mutation does not confer astroglial proneness to cell demise, at least for this experimental set up.

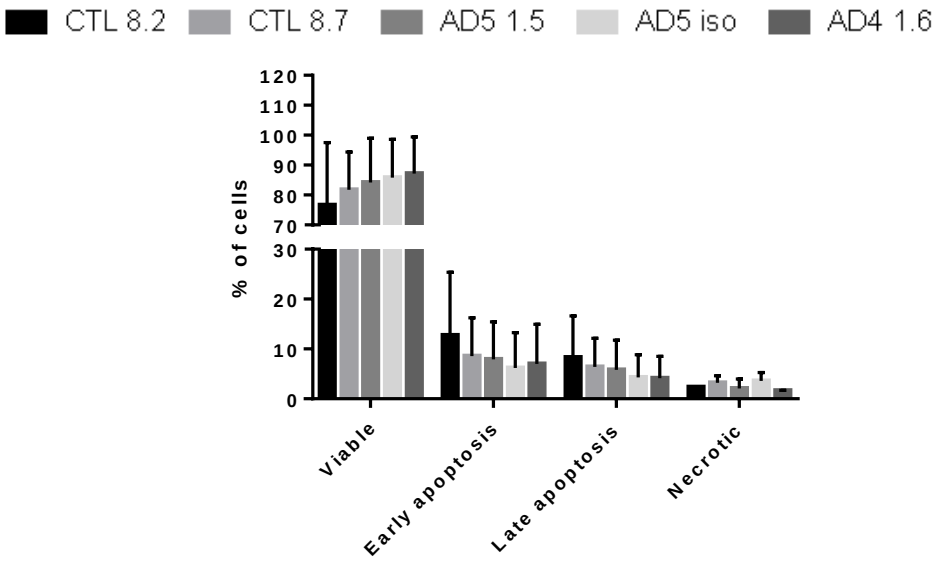

Figure III.13-Evaluation of cell viability in iPSCs-derived astrocytes from controls and AD patients, as well as from AD isogenic controls. Astrocytes differentiated from astrospheres of iPSCs cell lines, controls, AD patients or isogenic AD controls, were grown in culture for 7 days and then cell viability was measured using Guava Nexin® Reagent. Four populations of cells were distinguished: viable cells (annexin V-PE and 7-AAD negative), early-apoptotic cells (annexin V-PE positive and 7-AAD negative), cells in late stages of apoptosis or dead (annexin V-PE and 7-AAD positive) and necrotic/debries (annexin V-PE negative and 7-AAD positive). Results are percentage of cells ± SEM (n=3-5 per group).

3.2. Exosomes released by astrocytes derived from AD-iPSCs show different size populations

Having isolated the exosomes from the iPSCs-derived astrocytes, we first decided to characterize them in terms of particle size and concentration using the NTA technique. As indicated in Figure III.14A, astrocytes from iPSCs of AD patients release a decreased concentration of exosomes (0.28-fold) that was maintained in isogenic controls (0.21-fold). Although the average diameter of exosomes did not differ between conditions (Figure III.14B), a more detailed analysis of the number of particles with each diameter size revealed roughly differences (Figure III.14C). While astrocyte-derived exosomes from control-iPSCs showed one single population ranging from 120 to 240 nm and peaking around 180 nm, exosomes released by astrocytes from AD-iPSCs showed two main populations, one from 140 to 220 nm (peaking near 180 nm) and another from 250 to 350 nm (peaking near 280 nm). Astrocyte-derived exosomes from AD isogenic-iPSCs showed a heterogeneous population ranging from 140 to 400 nm in diameter size.

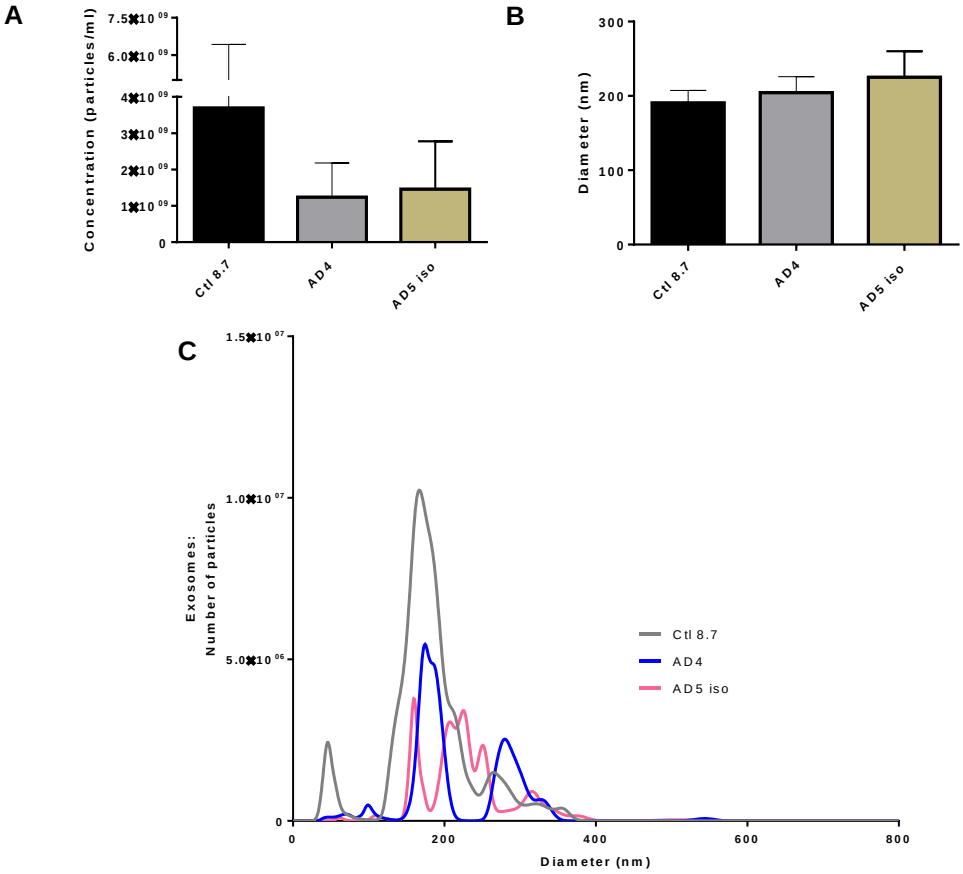

Figure III.14-Size and particle concentration of exosomes derived from astrocytes differentiated from iPSCs. Astrocytes differentiated from astrospheres of iPSCs cell lines, controls, AD patients or isogenic AD controls, were grown in culture for 7 days and then exosomes were isolated from culture media by ultracentrifugation. Exosomes characterization was performed by Nanoparticle Tracking Analysis (NTA) using the NanoSight. (A) Results of particle concentration are expressed as particles/ml. (B) Graph bars represent global results of averaged particle size expressed in nanometers (nm). (C) Histogram represent exosomes concentration (particles/ml) along diameter (nm) variation. Results of graph A and B are represented as mean (± SEM) from several readings of one independent experiment.

3.3. Astrocytes derived from iPSCs of AD patients show decrease of GFAP expression

Reactive astrocytes in addition to cytokines and inflamma-miRs production, also undergo cell hypertrophy with upregulation, for instance, of GFAP and S100B (Dzamba et al., 2016; Yi et al., 2016). These cell markers are widely used for identification of astrogliosis (Colombo and Farina, 2016). Noteworthy, GFAP, S100B and GLT-1 have been used to identify differentiated astrocytes from iPSCs (Kleiderman et al., 2016), reason why we assessed these markers in our astrocytes differentiated from iPSCs samples.

As shown in Figure III.15 only 50% of astrocytes differentiated from iPSCs and kept in culture for 7 days express GFAP, while almost 100% express S100B. Curiously, astrocytes differentiated from iPSCs of AD patients, namely the AD5, have a lower number of GFAP positive cells (~0.7-fold), when compared to all the others, while all GFAP positive cells are also positive for S100B.

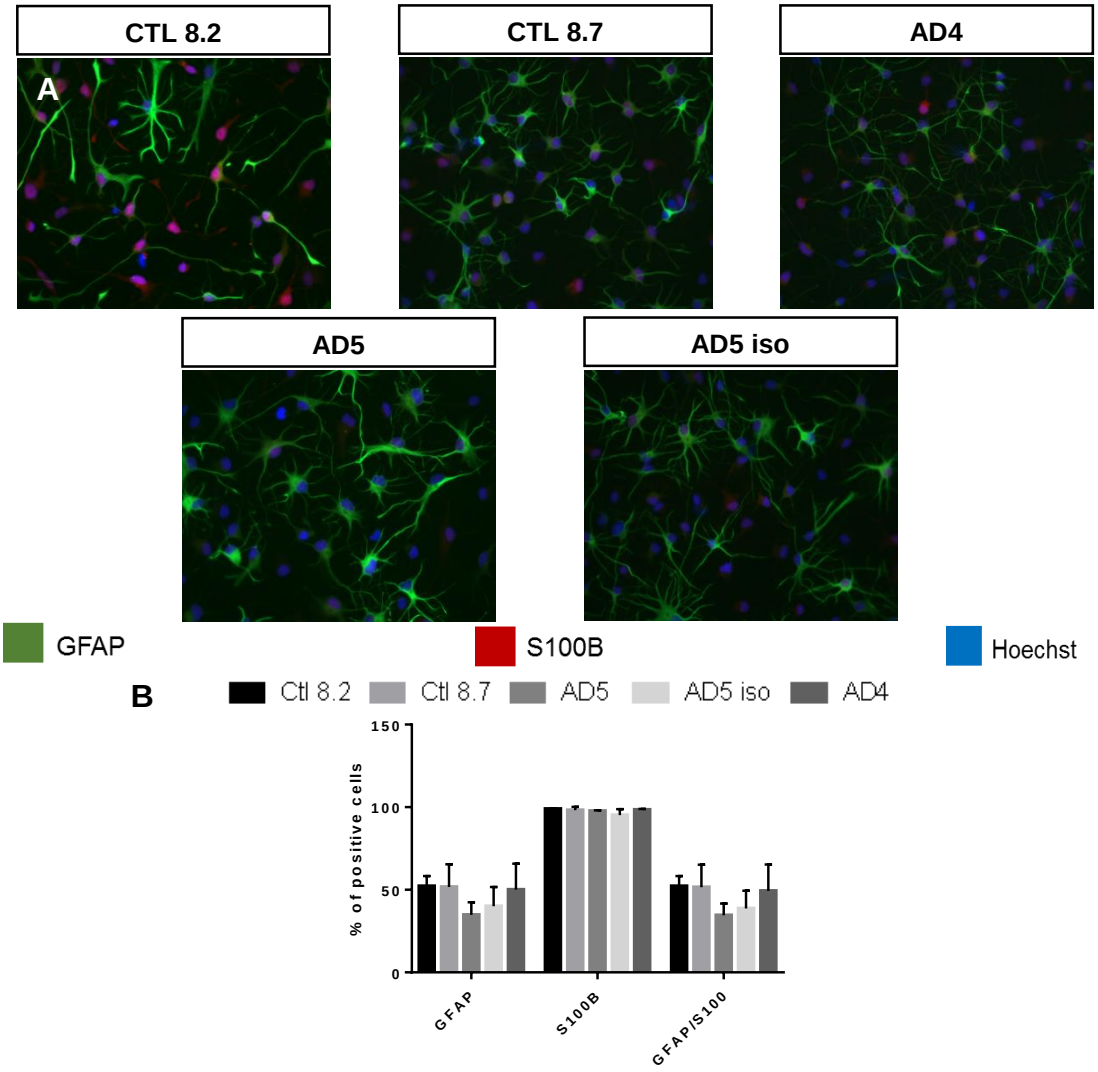

Figure III.15-GFAP marker is reduced in astrocytes derived from AD-iPSCs. Astrocytes differentiated from astrospheres of iPSCs cell lines, controls, AD patients or isogenic AD controls, were grown in culture for 7 days and then fixed. (A) GFAP and S100B were evaluated by double staining immunocytochemistry using mouse anti-GFAP and rabbit anti-S100B antibodies, followed by a fluorescent-labeled secondary antibody. Representative images of one experiment are shown. (B) Positive cells were counted using the ImageJ software and results are represented as mean (± SEM) of three independent experiments.

Concerning GLT-1 (Figure III.16), its presence was found in ~80% of total astrocytes differentiated from iPSCs that were kept in culture for 7 days. In this case, only the astrocytes differentiated from iPSCs of AD patient AD4, showed a higher number of GLT-1 positive cells (~1.2-fold), in comparison with the other cell lines. Once again, almost all GFAP positive cells also express GLT-1.

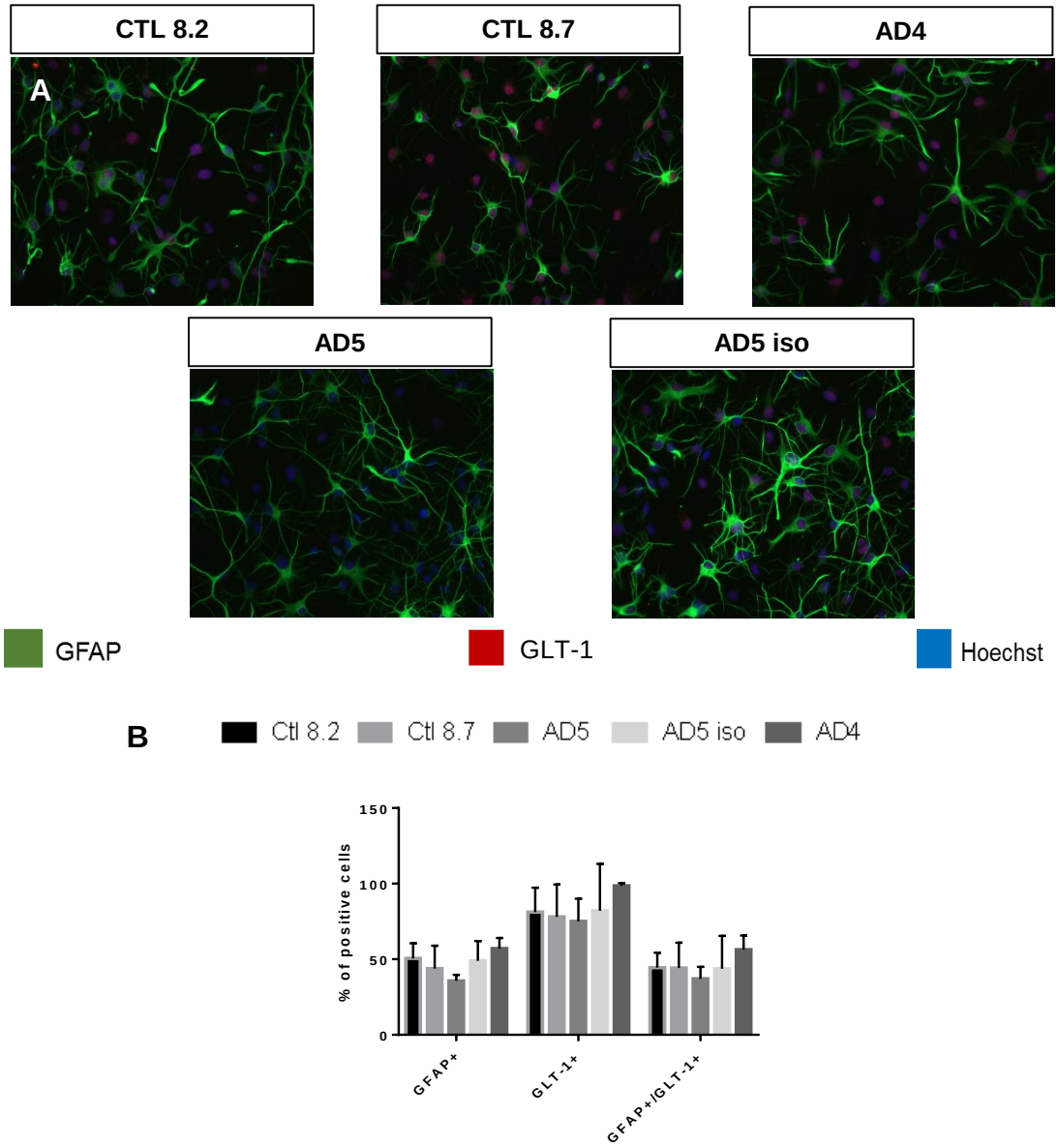

Figure III.16-GLT-1 marker is higher in astrocytes derived from AD-iPSCs of patient AD4. Astrocytes differentiated from astrospheres of iPSCs cell lines from controls, AD patients or isogenic AD controls, were grown in culture for 7 days and then fixed. (A) GFAP and GLT-1 were evaluated by double staining immunocytochemistry using mouse anti-GFAP and rabbit anti-GLT-1 antibodies, followed by a fluorescent-labeled secondary antibody. Representative images of one experiment are shown. (B) Positive cells were counted using the ImageJ software and results are represented as mean (± SEM) of three independent experiments.

3.4. iPSCs-derived astrocytes from patients with PSEN1ΔE9 mutation show a depressed RAGE/miR-155 pathway with impact in extracellular vesicle molecular cargo

Astrocytes have been pointed out as crucial regulators of neuroinflammation and its reactive activity may exacerbate inflammatory reactions and tissue damage (Colombo and Farina, 2016). On the other hand they also exert several neurotrophic functions that keep brain homeostasis, functions that have been reported to be impaired in AD models (Rodríguez-Arellano et al., 2016).

In Figure III.17A it is shown that, in terms of miRNAs expression, miR-155 was significantly reduced in astrocytes differentiated from iPSCs of AD patient AD5 relatively to astrocytes from controls (0.3-fold, $p<0.05$), observing a similar trend for AD4 (0.6-fold). However, miR-124, miR-21, miR-125b and miR-146a cell expression did not show significant differences in astrocytes differentiated from iPSCs of AD patients when compared to astrocytes from iPSCs of controls or respective isogenic cells (Figure III.17B,C,D,E). Such results led us to hypothesize that without facing an immunostimulatory stimulus, the generated astrocytes sustain essentially a steady state phenotype, although further studies are required to confirm or deny such hypothesis.

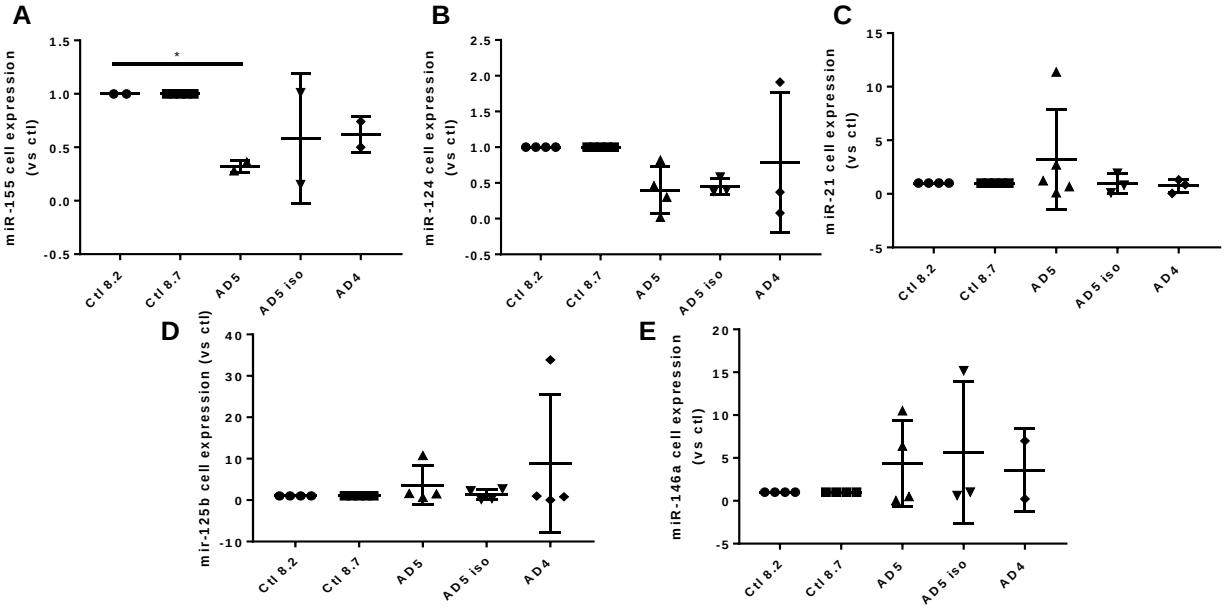

Figure III.17-MiR-155 is downregulated in astrocytes differentiated from AD-iPSCs of patient AD5. Astrocytes differentiated from astrospheres of iPSCs cell lines, controls, AD patients or isogenic AD controls, were grown in culture for 7 days and then RNA was isolated. miRNA expression in iPSCs derived astrocytes was evaluated by quantitative Real-Time PCR (qRT-PCR). Relative miR-155 (A), miR-124 (B), miR-21 (C), miR-125b (D) and miR-146a (E) levels. Results are represented as mean (± SEM) from at least three independent experiments and fold change determined vs control cells. *$p<0.05$ vs controls.

Next we evaluated the same miRNAs in the exosomes released by these astrocytes. Here, we could only detect a significant reduction of miR-146a in exosomes from astrocytes of iPSCs of AD5 patient when compared to exosomes from astrocytes of control cells (0.2-fold, $p<0.05$) (Figure III.18E). On the other hand, miR-124 (Figure III.18B) was significantly upregulated in exosomes from astrocytes

of iPSCs of AD5 isogenic control (39.9-fold, $p<0.01$), which may put in question the validity of this isogenic control cell line.

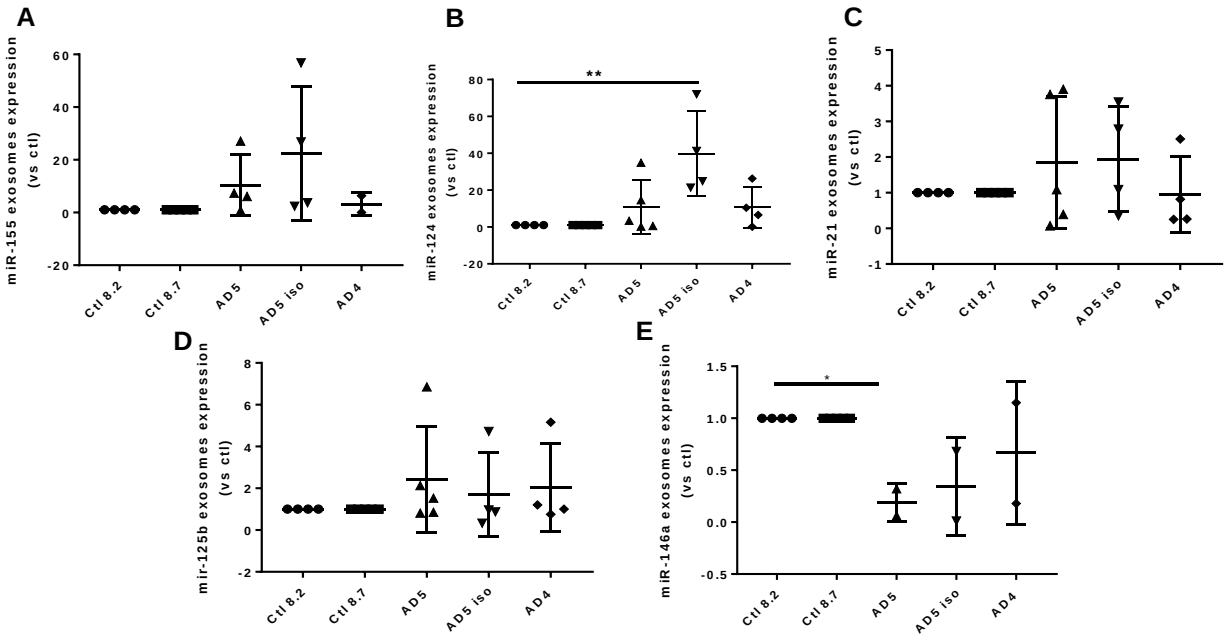

Figure III.18- Exosomes released by iPSCs-derived astrocytes show inflammatory-associated microRNA variability, with downregulation of miR-146a in those released by astrocytes derived from AD5-iPSCs and upregulation of miR-124 only in those from. Astrocytes differentiated from astrospheres of iPSCs cell lines from controls, AD patients or isogenic AD controls were grown in culture for 7 days and then exosomes were isolated and RNA extracted. miRNA expression in iPSCs-derived exosomes was evaluated by quantitative Real-Time PCR (qRT-PCR). Relative miR-155 (A), miR-124 (B), miR-21 (C), miR-125b (D) and miR-146a (E) exosomes levels. Results are represented as mean (± SEM) from at least three independent experiments and fold change determined *vs* exosomes from control cells. *$p<0.05$ and **$p<0.01$ *vs* controls.

In terms of inflammatory related molecules, we decided to evaluate also the alarmins S100B and HMGB1 and respective receptor RAGE, as well as the cytokines involved in an inflammatory event TNF-α and IL-10. As shown in Figure III.19C, there is high variability between different experiments and no significant changes in the expression of S100B, as expected by the immunostaining data, or HMGB1 in astrocytes of iPSCs of AD patients when compared to matched controls. However, RAGE expression was significantly downregulated in astrocytes derived from iPSCs of AD5, AD4 cells and also of AD 5 isogenic cells relatively to controls (0.22, 0.17 and 0.15-fold, respectively, $p<0.01$). This finding suggests that these cells have decreased ability to bind S100B and HMGB1 and possibly even Aβ, thus indicating a decreased ability to mount a reactive response, at least in our experimental conditions. Concerning the cytokines TNF-α and IL-10 (Figure III.19 D,E), given the high variability between experiments there was no significant changes observed between astrocytes derived from iPSCs of controls or AD patients, corroborating once again the lack of astrocyte reactive phenotype in cells from AD patients when they are not stimulated.

Since Cx43 is the major gap junction protein of astrocytes (Brand-Schieber et al., 2005) and reported to be increased at sites of amyloid plaques in AD (Nagy et al., 1996), we also evaluated the mRNA expression of this molecule. As described in Figure III.19F, no changes were observed in Cx43 expression between the astrocytes derived from the different iPSCs.

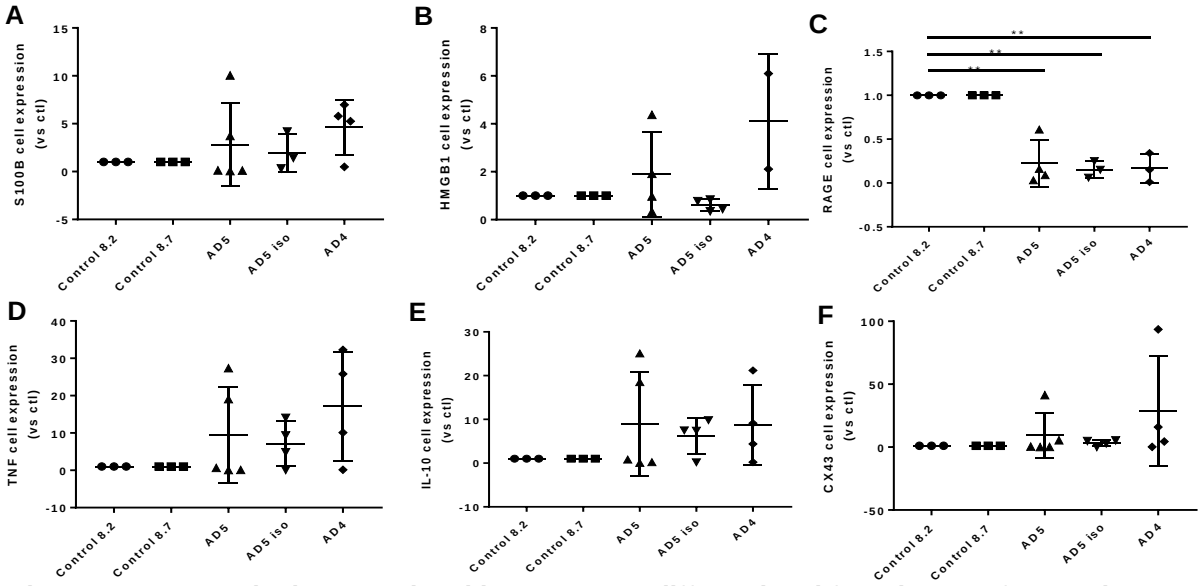

Figure III.19-RAGE is downregulated in astrocytes differentiated from iPSCs of AD patients and isogenic control. Astrocytes differentiated from astrospheres of iPSCs cell lines from controls, AD patients or isogenic AD controls were grown in culture for 7 days and then RNA extracted. mRNA expression in iPSCs derived astrocytes was evaluated by quantitative Real-Time PCR (qRT-PCR). (A) Relative S100 calcium-binding protein B (S100B), (B) High-mobility group box 1 (HMGB1), (C) Receptor for advanced glycation end-products (RAGE), (D) Tumor necrosis factor-α (TNF-α), (E) Interleukin-10 (IL-10) and (F) Connexin 43 (Cx43) levels. Results are represented as mean (± SEM) from at least three independent experiments and fold change determined vs control cells. ***p*<0.001 *vs* controls.

Interestingly, when we evaluated the expression of such inflammatory-related mediators in the exosomes released by these iPSCs-derived astrocytes (Figure III.20), we observed a downregulation of S100B (AD5, 0.16-fold, *p*<0.001; AD4, 0.05-fold, *p*<0.001), HMGB1 (near 0-fold for AD5 and AD4, *p*<0.001) and TNF-α (AD5, 0.42-fold, *p*<0.001; AD4, 0.02-fold, *p*<0.001) when compared to exosomes from controls. Finally, we decided to evaluate Cx43 mRNA expression, since it has been described that exosomes carrying Cx43 are able to exchange molecules with recipient cells that also express this molecule through gap junction channels (Varela-Eirin et al., 2017). Nevertheless, we did not find any change, except a downregulation tendency for the Cx43 gene in exosomes from astrocytes generated from isogenic AD5-iPSCs.

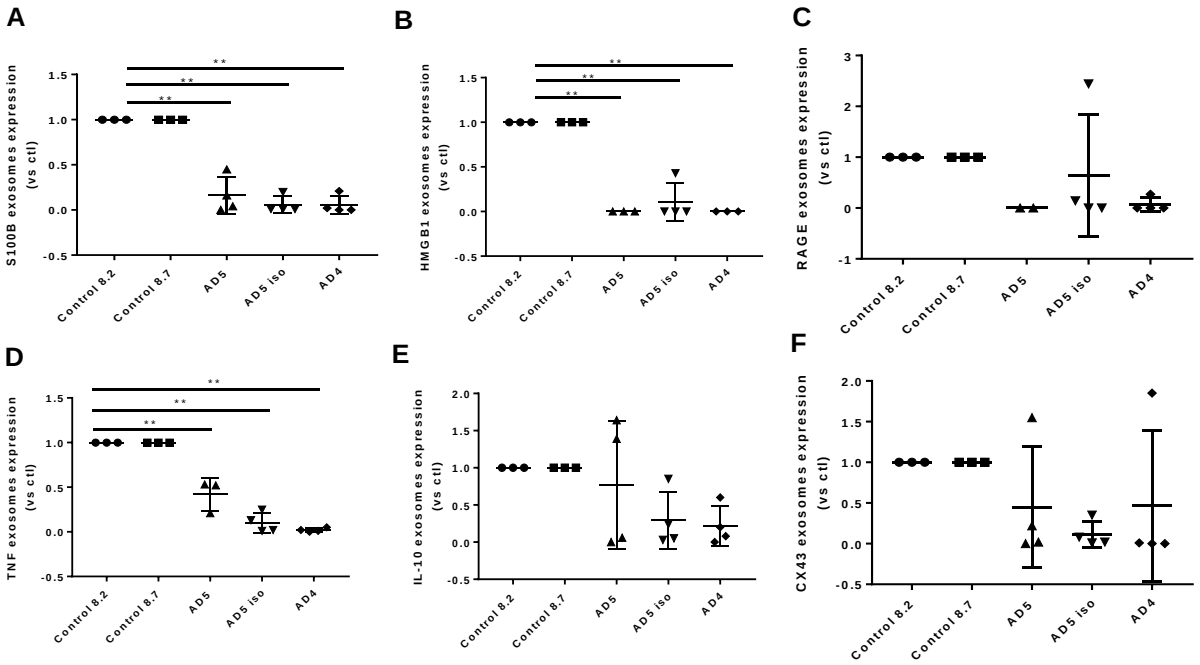

Figure III.20-Exosomes derived from astrocytes differentiated from iPSCs of AD patients show decreased levels of S100B, HMGB1 and TNF-α. Astrocytes differentiated from astrospheres of iPSCs cell lines from controls, AD patients or isogenic AD controls were grown in culture for 7 days and then exosomes were isolated and RNA extracted. mRNA expression in iPSCs derived astrocytes from respective exosomes was evaluated by quantitative Real-Time PCR (qRT-PCR). (A) Relative S100 calcium-binding protein B (S100B), (B) High-mobility group box 1 (HMGB1), (C) Receptor for advanced glycation end-products (RAGE), (D) Tumor necrosis factor-α (TNF-α), (E) Interleukin-10 (IL-10) and (F) Connexin 43 (Cx43) levels. Results are represented as mean (± SEM) from at least three independent experiments and fold change determined vs exosomes from control cells. ***p*<0.001 *vs* controls.

Overall our results indicate that astrocytes differentiated from AD-iPSCs display a less reactive phenotype and release exosomes depleted in alarmins and cytokines.

IV. Discussion

AD is currently the leading cause of dementia in the elderly and it is one of the most critical public health problems in terms of social and political impacts. Therefore, great efforts have been made by the scientific community in order to acquire new insights to unveil AD pathogenesis. Over the time, increasing evidences suggest that AD is not a cell autonomous disease, by also including strong interactions with immunological mechanisms in the brain. Neuroinflammation and cell interaction emerge as promising sources of targets that may be valuable to generate novel therapies and improve AD diagnosis.

Microglia and astrocytes are involved in disease severity and progression by exacerbation of the inflammatory response. However, their role in AD is still not well clarified and remains a "hot topic" for researchers. Additionally, EVs released by neurons and glial cells contribute to AD pathogenesis, as they may serve as vehicles for cell activation factors and disease spreading.

Animal cell models have been the main model for the majority of the studies underlying AD pathogenesis mechanisms. However, when it comes to extrapolate data from animals to human cells a huge controversy raises up. Many studies on AD pathogenesis were already performed by the use of human immortalized cell lines derived from cancer, but the lack of resemblance with functional cells is still a major problem. In order to solve this problematic issue, researchers have focused on the possibility to create new *in vitro* models that can truly recapitulate AD pathogenesis. iPSCs are pointed as a novel approach and due to their tremendous potential, they may fill the gap between *in vitro* studies and the human body.

In the first part of this study, we used the CHME3 microglia cell line to explore the response of human microglia to exosomes derived from the SH-SY5Y APP_{Swe} cells, our *in vitro* experimental AD model, when compared to control SH-SY5Y cells. Our particular interest was to assess changes in microRNA profile and inflammatory phenotype. In the second part, we used astrocytes generated from iPSCs derived from AD patients, again to investigate changes in the microRNA profile, as well as inflammatory phenotype characteristics, when compared to astrocytes derived from iPSCs of healthy individuals or to the respective AD isogenic cell line.

Previous data from our group showed that SH-SY5Y APP_{Swe} cells express increased concentration of APP and secrete significant elevated levels of $A\beta_{1-40}$, when compared to SH-SY5Y cells. We started by differentiating the neuroblastoma cell line for 9 DIV using RA, following cell collection and exosome purification from the extracellular media by differential centrifugation. Isolated particles showed a distribution around ~180 nm, which is compatible with exosomal formation (Park et al., 2015). Looking at the cellular and exosomal content, we noticed a major representation of miR-124, miR-155, miR-146a, miR-21 and miR-125b in SH-SY5Y APP_{Swe} cells and of miR-124, miR-125b and miR-21 in their respective exosomes, in comparison with SH-SY5Y cells. In fact, miR-124 has been found to be the most abundant microRNA expressed in CNS, namely in neurons (Lagos-Quintana et al., 2002). In an AD model, the inverse relationship between miR-124 and BACE1 was demonstrated (Fang et al., 2012a). Thus, with increased levels of miR-124 in SH-SY5Y APP_{Swe} cells we should expect a reduced expression of BACE1 levels in these cells. However, previous results from the group showed a marked

release of $A\beta_{1-40}$ to the extracellular media indicating an active BACE1. Moreover, miR-125b has been reported as an important regulator of neuronal differentiation (Le et al., 2009) and has been implicated in AD pathogenesis (Banzhaf-Strathmann et al., 2014). In accordance, we observed a marked increase in both SH-SY5Y APP_{Swe} cells and in their derived exosomes. On the other hand, miR-155 and miR-146a are associated with inflammatory processes (Cardoso et al., 2012; Lukiw et al., 2013). While miR-155 was described to be upregulated in AD triple transgenic mouse model (Guedes et al., 2014), which was also verified in our lab for as early as 3-month-old animals. In addition, miR-146a was found increased in serum of early MCI patients, but decreased in late AD patients (Dong et al., 2015). So, our results showing increased levels of both miRNAs suggest that this model may mimic an early phase of the disorder. MiR-21 role is associated with inflammation resolution (Ponomarev et al., 2013), however, when part of EV cargo it can trigger inflammatory processes via TLR signaling (Yelamanchili et al., 2015). Thus, it is possible that SH-SY5Y APP_{Swe}-derived exosomes with increased miR-21 may be responsible for the CHME3 activation observed. Concerning inflammatory-related molecules, a major representation of S100B mRNA alarmin in both SH-SY5Y APP_{Swe} and respective exosomes was noticed, in comparison with data from SH-SY5Y cells. On the contrary, HMGB1, TNF-α and IL-10 mRNAs were observed in SH-SY5Y APP_{Swe} cells, but not in their derived exosomes. Curiously, increased miR-21 and IL-10 interaction have been related to inflammation resolution (Ponomarev et al., 2013). MicroRNAs and inflammatory molecules increased presence in exosomes is not without precedent due to their endosomal origin, and as a result of inward budding during the formation of the exosomes, reason why it is considered that exosomes partially recapitulate the cell of origin (Rabinowits et al., 2009; Simons and Raposo, 2009). However, we must not forget that we evaluated mRNA expression and not protein expression, since we could not obtain enough yield from the isolated exosomes to perform a Western Blot analysis. So, it may be reasonable to assume that, if we assessed exosomal protein content we could have found these cytokines.

After characterizing neuroblastoma-derived exosomes and their inflammatory cargo, we evaluated the response of healthy human CHME3 microglia when exposed to exosomes from SH-SY5Y APP_{Swe} cells, as compared to those from SH-SY5Y cells. Microglia response was evaluated after a short period of exposure to exosomes (24 h), and after a 24 h period of recovery without exosomes, at 48 h final incubation. We confirmed that exosomes from both neuroblastoma cell lines were incorporated by microglial cells, and we then assessed microglia function/dysfunction by lysosome activity, autophagy and cell viability as early effects determined by exosomes. Lysosomes are the key digestive organelles of the cell and can digest a wide variety of substances, including misfolded proteins, like Aβ. Microglia are crucially involved in phagocytosis and degradation of Aβ (Lee and Landreth, 2010). However, some reports suggest that microglia is incapable of digest engulfed Aβ, since the lysosomal acidification is inadequate for the digesting process, thus strongly suggesting the involvement of lysosomal dysfunction in the impairment of Aβ clearance (Majumdar et al., 2007). Furthermore, defective autophagy was observed in an *in vivo* AD model, additionally suggesting that lysosomes dysfunction was involved in autophagic impairment (Wolfe et al., 2013). Exosomes and their involvement in Aβ release have already been described in *in vitro* AD models. Additionally, an exosomal-associated protein, Alix, was shown to be specifically enriched in amyloid plaques of AD brain sections, indicating a novel role for exosomes in

AD pathogenesis (Rajendran et al., 2006). In our results, we observed that lysosomal and autophagic activities were compromised in CHME3 microglia treated with exosomes from SH-SY5Y APP_{Swe} cells. Indeed, at 48 h after initial incubation a significant decrease in the intensity of both lysosomal (LysoTracker) and autophagic (LC3) markers was observed. Nevertheless, we also observed loss of LC3 staining in CHME3 cells exposed to SH-SY5Y exosomes when compared to non-treated CHME3 cells, suggesting that this phenomenon may be produced by exosome exposure. Next, we evaluated whether exosomes from the AD model could also compromise CHME3 microglia cell viability. A few studies have focused on exosomes as having a role on cell death (Dehbashi Behbahani et al., 2016). In cancer, some studies reported the impact of exosomes on cancer cells wherein it is suggested that exosomes released by pancreatic cancer cells are able to stimulate apoptosis via mitochondrial pathways (Pigati et al., 2010). It has also been proposed that the lipid profile of exosomes (e.g. cholesterol) determines the activation of caspase-3 and caspase-9 (Dehbashi Behbahani et al., 2016). In our results, we observed that microglia cell viability was slightly compromised when exposed to SH-SY5Y APP_{Swe}–derived exosomes at the end of 48 incubation, with a significant difference from cells exposed to SH-SY5Y-derived exosomes. However, these levels showed to not be enough to compromise the evaluation of the CHME3 microglia reactive response.

Next, we focused on inflammatory microglia response towards the presence of exosomes, since neuroinflammation has been known as a central feature of AD pathogenesis, and microglia a key cellular mediator of the neuroinflammatory processes (Streit et al., 2004; Chen et al., 2016). To verify the existence of neuroinflammation, we dissected the effects produced by exosomes on microglia expression of inflamma-miRs and inflammatory-related molecules in an attempt to better clarify the role of neuron-microglia communication. First, we analyzed CHME3 microRNA profile in response to exosomes from both SH-SY5Y and SH-SY5Y APP_{Swe} cells. Mir-155 is one of the most well studied immune-related microRNA in AD associated to neuroinflammatory events (Guedes et al., 2014). It was already described a strong upregulation of miR-155 in the brain of young animals, simultaneously with the increase of microglia activation before the appearance of extracellular Aβ aggregates, suggesting a key role of miR-155 in early neuroinflammation (Guedes et al., 2014). In our study, we observed only slightly elevated levels of miR-155 at 24 h and 48 h when compared to CHME3 treatment with exosomes from SH-SY5Y cells. It deserves to be noticed that at 24 h, exosomes collected from microglia incubation media may derive from both microglia and remaining neuroblastoma-derived exosomes not captured by microglia. However, at 48 h, since we replaced microglia medium, exosomes are predominantly derived from CHME3 microglia and hypothetically from microglia processed neuroblastoma-derived exosomes. Since miR-155 levels were elevated at SH-SY5Y APP_{Swe}-derived exosomes, the observed response at 24 h may be a cumulative effect of both neuroblastoma exosomes and microglia response.

Interestingly, we saw that microglia downregulated the expression of miR-124 upon exposure to exosomes, independently of their origin. In fact, Pinto and colleagues, in an *in vitro* ALS model showed that microglia exposed to exosomes from motor neurons exhibited a downregulation of miR-124 (Pinto et al., 2017). This could be translated to microglia activation, reinforced by the slight increase of miR-155 expression already discussed. Actually, miR-124 is thought to keep microglia in a quiescent state and its expression decreases when microglia is activated (Ponomarev et al., 2011). Finally, we saw that

miR-21 expression seemed to increase in CHME3 treated with exosomes from SH-SY5Y APP_{Swe} cells. MiR-21 was demonstrated to play an important role in inflammation resolution by triggering the expression of pro-inflammatory IL-10. In the other hand, miR-21 can act as an inflammatory mediator by binding TLR7 and TRL8 (Liu and Abraham, 2013). This dual role is determined by many factors, such as local concentration of extracellular miR-21, and by the kinetics of mRNA targets associated with intracellular miR-21 levels (Liu and Abraham, 2013). Thus, the upregulation of miR-21, although not significant, may indicate either an attempt of microglia to return to its steady state, or an inflammatory response to exosomes derived from SH-SY5Y APP_{Swe} cells. These findings are also translated in CHME3 derived exosomes, wherein exosomes from CHME3 treated with exosomes from the SH-SY5Y APP_{Swe} cells significantly contained higher levels of miR-21 than exosomes from CHME3 treated with SH-SY5Y-exosomes. MiR-21 was described as having a toxic effect when released in exosomes (Yelamanchili et al., 2015). Such results could be related to cell-to-cell communication mediated by exosomes leading to CHME3 activation, which later may sense other surrounding cells.

After studying microglial inflamma-microRNA profile in response to exosomes released by the two neuroblastoma cell lines, an additional goal was to study microglia response to exosomes in terms of alarmins and inflammatory-related molecules. Microglia express a plethora of cell surface receptors that are used to detect foreign material. Engagement of microglia defense mechanisms incites the conversion of a surveilling microglia to an activated phenotype associated with the upregulation of inflammatory genes and secretion of cytokines, chemokines and acute phase proteins (Cameron and Landreth, 2010). S100B has been described as a primary astrocytic protein; however, it is also released by damaged cells or necrotic cells (Sorci et al., 2010). Thus S100B is considered a strong marker of brain injury and increased levels of S100B have been found in AD patients (Sen and Belli, 2007). Another alarmin that we also determined was HMGB1. Release of HMGB1 into the extracellular space also represents a sign of cell injury (Szabo and Hoffman, 2012). Therefore, these two alarmins have been classified as important mediators of neuroinflammation in AD (Fang et al., 2012b). Our results indeed showed an upregulation of S100B and HMGB1 in the CHME3 microglial cells treated with the SH-SY5Y APP_{Swe}-derived exosomes, when compared with the matched control, at both 24 and 48 h, reflecting microglia stimulation by exosomes derived from mutated cells. An interesting finding was that exosomes from CHME3 cells did not contain HMGB1 mRNA. This molecule was described as being predominantly secreted in soluble fractions (Davalos et al., 2013), suggesting that it will be important to later assess the HMGB1 protein not only in exosomes, but also in the extracellular medium. Interaction of S100B and HMGB1 with the receptor RAGE results in the activation of NF-κB and the recruitment of inflammatory cells, which in turn amplifies the process of tissue damage (Chavakis et al., 2004). Actually, together with upregulation of S100B and HMGB1 mRNA expression, we saw that RAGE suffered upregulation in CHME3 treated with exosomes from SH-SY5Y APP_{Swe} cells, when compared with CHME3 treated with SH-SY5Y-derived exosomes. Microglia expression of pro-inflammatory cytokines like TNF-α was upregulated when in the presence of exosomes from the mutated cells, reinforcing microglia activation. Simultaneous elevation of IL-10 gene expression could mean that the population of microglial cells became heterogeneous after the stimulation with exosomes from SH-SY5Y APP_{Swe} cells. Indeed, in another study from our group it was showed that microglia exposed to exosomes from

motor neurons with an ALS mutation evidence upregulated IL-10 expression, together with a set of M1 and M2 polarization markers sustaining the presence of a mixed population of microglial cells, probably as an attempt to moderate the inflammatory phenotype (Pinto et al., 2017). Finally, exosomes from CHME3 cells treated with SH-SY5Y APP_{Swe}-derived exosomes did not quite recapitulated the M1 polarized microglia profile. In a study conducted by Konadu, where an association between cytokines and exosomes released by HIV-seropositive patients was described, TNF-α cytokine was upregulated in exosomes from patients (Konadu et al., 2015). We observed an increase in the expression of TNF-α mRNA in exosomes from the microglia treated with neuron-derived exosomes, but significance was not achieved. It would be interesting in the future to investigate whether the cytokine is more represented than the mRNA in the exosomal cargo.

The final goal of this thesis was to evaluate whether non-stimulated hiPSCs-derived astrocytes from AD patients expressing the PSEN1ΔE9 mutation showed a distinct reactive phenotype, when compared to matched controls. Astrocytes are the main homeostatic cells in CNS and their dysfunction may be implicated in AD progression. Moreover, astrocytes and microglia have gain special attention due to their contribution to non-cell autonomous mechanisms in a variety of neurodegenerative diseases (Brites and Vaz, 2014; Meyer and Kaspar, 2016). Here, the main focus was to better understand if astrocytes from AD patients express a reactive phenotype due to a specific mutation involved in FAD pathogenesis. Some recent studies have focused on astrocytes immunocompetency-associated stimulus in pathogenesis of neurodegenerative disorders and neuroinflammation (Santos et al., 2017). However, non-stimulated astrocytes remain poorly understood. Jones and colleagues described that astrocytes derived from FAD and SAD patients are smaller, less heterogeneous and exhibit almost complete absence of processes, when compared with healthy induced astrocytes. Here, either AD astrocytes, isogenic cells or healthy controls expressed less GFAP and GLT-1 than S100B, while GFAP positive cells were decreased and GLT-1 positive ones increased in one AD cell line. Moreover, it was described that FAD and SAD astrocytes display compromised constitutive secretion of pro-inflammatory cytokines (Jones et al., 2017). Astrocytic microRNA profile in neurological conditions has shown some diversity. For example, while upregulation of miR-21 in SCI has been implicated in astrocyte hypertrophy and glial scar progression (Bhalala et al., 2012), miR-125b was shown to be absent in astrocytes from deeper grey matter (Rao et al., 2016b). On the other hand, miR-155 and miR-146a upregulation has been implicated in AD pathogenesis, or reported under chronic stimulation with the presence of inflammatory molecules (e.g. IL-1β, IL-6, TNF-α and HMGB1) (Iyer et al., 2012; Guedes et al., 2014). Moreover, receptors on astrocyte surface mediate the production of pro-inflammatory molecules (e.g. TNF-α) under a pathological stimulus (Calsolaro and Edison, 2016). Our results showed that astrocytes derived from AD patients evidence a less reactive phenotype with significant decrease of the pro-inflammatory miR-155 and downregulation of the RAGE receptor, relatively to controls. We also saw that AD astrocytes showed two populations of exosomes instead of the single population detectable in controls. Lastly, exosomes derived from astrocytes differentiated from AD-iPSCs showed decreased levels of S100B, HMGB1 and TNF-α, not supporting the dissemination of these genes via exosomes. Taken together, these results suggest that AD astrocytes display a less reactive phenotype and lead to exosomes depleted of inflammatory machinery. It should be emphasized that these astrocytes represent

a simplified model of AD, whereby additional interactions with other cells or other extracellular components could influence the astrocytic phenotype (Jones et al., 2017).

Concluding Remarks

In summary, the results obtained indicate the existence of upregulated inflammatory microRNAs, alarmins, RAGE receptor and cytokines in SH-SY5Y APP_{Swe} cells and that their exosomes contain elevated miR-155, miR-21 and S100B gene expression, as schematically represented in Figure IV.1. Such exosomes are collected by microglial cells and determine an acute inflammatory response after 24 h incubation, with the production of pro-inflammatory cytokines and microRNAs related to inflammation. Additionally, microglia revealed to be unable to restore their steady state phenotype, once the production of pro-inflammatory cytokines and inflamma-microRNAs was maintained at the end of the 24 h additional recovery period, i.e. at 48 h. Treated microglia showed to be dysfunctional with the impairment of lysosomal and autophagic activities. Therefore, we may summarize that exosomes from SH-SY5Y APP_{Swe} contribute to: (i) early over-reaction of microglia and, consequently, to neuroinflammation; (ii) impair microglia function, while preventing their restoration into the surveilling phenotype, thus compromising their role in the maintenance of cellular hemostasis.

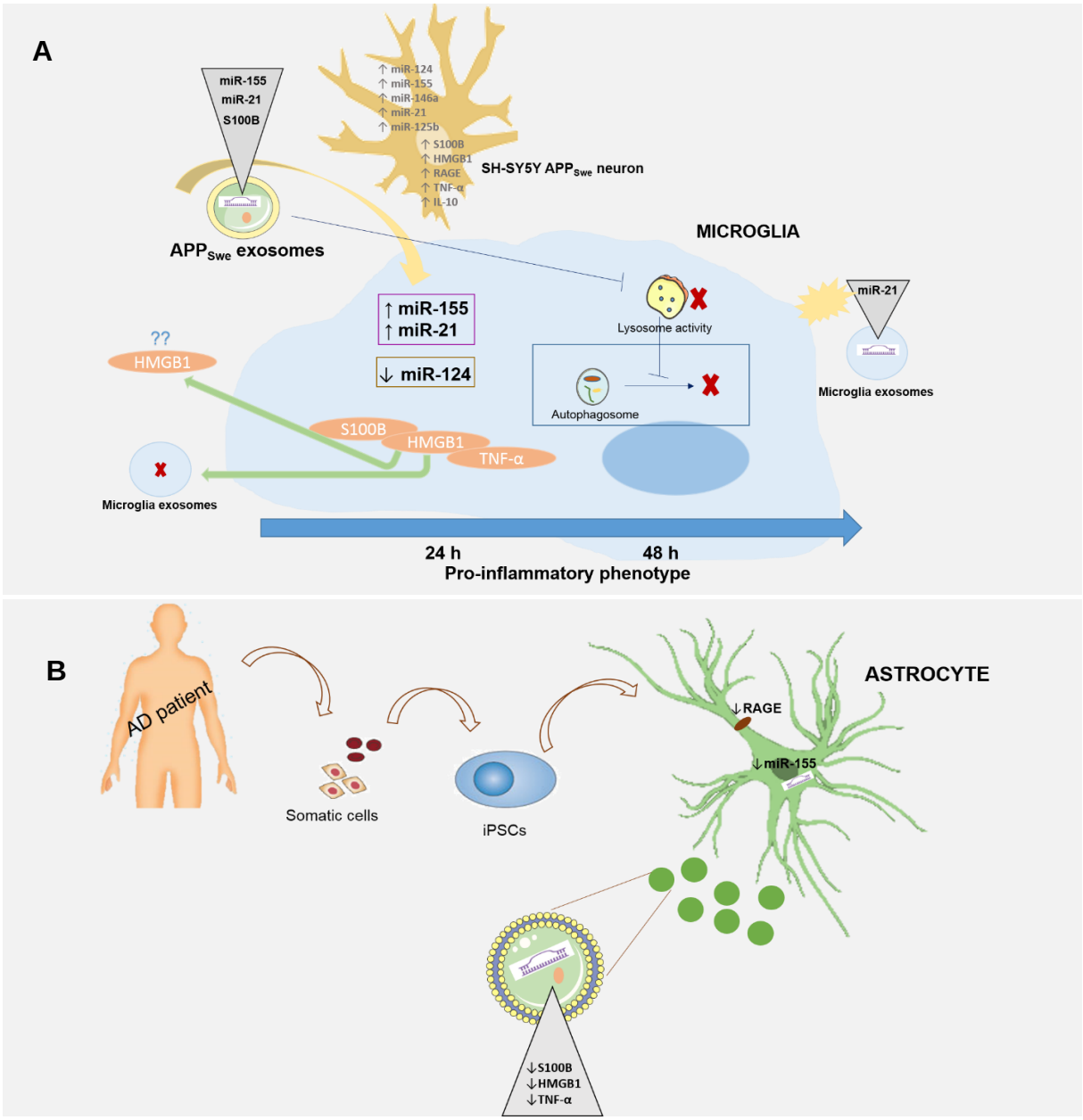

Figure IV.1-Schematic representation of the main research contributions of this thesis. (A) Key features of microglia response to exosomes released by SH-SY5Y APP$_{Swe}$ cells (AD neurons). Upregulation of inflammatory-associated mediators, including microRNAs (miR-155/miR-124/miR146a/miR-21/miR-125b), alarmins (S100B/HMGB1), RAGE receptor and cytokines (TNF-α/IL-10) was observed in AD neurons. From those, miR-155, miR-21 and S100B were transferred into AD neuron-derived exosomes that are captured by microglia and co-localize with lysosomes, increasing their activation and subsequent exhaustion, ultimately contributing to a minor, although significant, loss of cell viability. AD-exosomes did not trigger alterations in microglia miRNA profile but determine increased expression of miR-21 after 24 h incubation and at the end of an additional period of 24 h for recovery, when compared to the effects produced by the exosomes from SH-SY5Y neurons. Interestingly, AD-exosomes were able to activate microglia by inducing overexpression of S100B, HMGB1, RAGE receptor, TNF-α and IL-10 genes, once they were not significantly upregulated in exosomes from AD-neurons. To note, however, that some, as HMGB1, may have been transferred into microglia as soluble factors. **(B) Characterization of the phenotype of astrocytes generated from AD-iPSCs and their derived exosomes.** Astrocytes from AD-iPSCs revealed to contain downregulated miR-155 and RAGE gene expression, and their derived exosomes decreased HMGB1, TNF-α and S100B mRNA levels. S100B, S100 calcium-binding protein B; HMGB1, High-mobility group box 1; RAGE, Receptor for advanced glycation end-products; TNF-α, Tumor necrosis factor-α.

As final note, we consider that our findings in CHME3/SH-SY5Y exosomes culture provide a drug-testing platform aimed at using exosome-based delivery technologies to modulate excessive microglia activation and astrocyte reactivity. Indeed, previous studies have reported the tremendous impact of cell-to-cell communication on glial cells activation and pathogenesis, supporting the urgent need of developing effective therapies that ultimately can be driven by exosomes. Indeed, exosomes have the potential to serve as a non-invasive intervention, while they are easily preserved, have lower immunogenicity and show capacity to cross the blood-brain barrier. Exosomes may have a very promising impact in preventing the dissemination of pathogenic proteins or molecules, as well as miRNAs, to other cells, avoiding propagation of toxicity and neuroinflammation, and in delivering therapeutic agents to target cells, in order to provide cell protection.

Our results obtained in astrocytes derived from AD-iPSCs demonstrate their depressed reactive profile in the absence of an immunostimulatory effect and corroborate that non-autonomous cell pathogenesis should be considered in AD. The model has been indicated to better mimic the pathophysiological mechanisms of AD with the advantage of allowing studies in *in vitro* models, whereby cell-to-cell communication impairment is described to play a major role in neurodegenerative processes. More important, human iPSCs are a new reality in the study of AD SAD and FAD pathogenesis, while holding a hope for regenerative and precision medicine.

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